Siderophilin Metal Coordination. Difference Ultraviolet Spectroscopy of Di-, Tri-, and Tetravalent Metal Ions with Ethylenebis[(*o*-hydroxyphenyl)glycine]†

Vincent L. Pecoraro, Wesley R. Harris,‡ Carl J. Carrano,§ and Kenneth N. Raymond*

ABSTRACT: So that the metal ion coordination site in the human iron transport protein, transferrin, could be probed, the complexation of a series of metal ions by the chelate analogue ethylenebis[(*o*-hydroxyphenyl)glycine] (EHPG) has been studied by difference UV spectroscopy, in which $\Delta\epsilon$ values per coordinated phenol have been determined for the metal complex vs. the protonated form of the ligand. With the exception of the Cu^{2+} complex, maxima are observed at 242 and 290 nm with a minimum at 269 nm. The $\Delta\epsilon$ values at 242 nm fall into two groups. Complexes of divalent metal ions (Zn^{2+}, Cu^{2+}, and Cd^{2+}) have $\Delta\epsilon$ values ranging from 5000 to 6600 M^{-1} cm^{-1} whereas larger $\Delta\epsilon$ values are observed for complexes of tri- and tetravalent metal ions (Th^{4+}, Ga^{3+}, Fe^{3+}, Ho^{3+}, Eu^{3+}, Er^{3+}, Tb^{3+}, and VO^{2+}), 7400–8700 M^{-1} cm^{-1}. It is known that the transferrin binding sites contain tyrosyl residues, but there has been considerable debate concerning the precise number of tyrosine groups which bind to specific metal ions. Since it has been the common practice to assume that the $\Delta\epsilon$ values for coordination by all metal ions are identical, the larger range of $\Delta\epsilon$ values actually observed here shows that such an assumption can actually lead to an erroneous tyrosine/metal site ratio. The difference spectra of transferrin and EHPG complexes are very similar, and we have taken the $\Delta\epsilon$ values of the EHPG complexes as estimates for the intrinsic $\Delta\epsilon$ for coordination of a single tyrosine ligand. The number of tyrosines bound per metal ion is then calculated on the basis of previously reported *total* $\Delta\epsilon$ values of several dimetallotransferrin complexes. The results show that two tyrosines are coordinated per metal ion for all the transition metals and the smaller lanthanides. Very large metal ions have difficulty fitting into one of the binding sites, and the number of coordinated metal ions decreases. This differential ability to coordinate large metal ions lends further support for nonequivalent complexation by the two metal binding regions of transferrin. A model for the Fe^{3+} transferrin binding site which is consistent with both these results and previous proton release and chemical modification studies is proposed in which a carbonato, a hydroxo, two tyrosyl, and two histidyl ligands are bound to the ferric ion to form a six-coordinate complex. It is further proposed that the smaller number of protons released upon binding of divalent ions such as Cu^{2+} is due to the effective replacement of the hydroxo group by a water molecule, due to the lower acidity of the dication aquo complexes.

Human serum transferrin,[1] the protein responsible for iron transport in the blood, possesses two inequivalent metal binding sites per molecule (Chasteen, 1977; Harris, 1977), which may have different physiological functions (Fletcher & Heuhns, 1968). There has been some controversy as to the nature and the number of ligands which coordinate ferric ion at each site. Some workers believe that the metal binding region is rich in a variety of ligands which may effectively coordinate many different metals (Chasteen, 1977). Histidine residues have been implicated in the binding process as well as carbonate and water (Koenig & Schillinger, 1969; Rogers et al., 1977; Zschocke et al., 1972; Buttkus et al., 1965; Ford-Hutchinson & Perkins, 1970). It has been well established that tyrosines are bound to the metal ion. However, whether two or three of these residues per site are involved in coordination is still debated (Gelb & Harris, 1980; Luk, 1971; Tan & Woodworth, 1969; Aasa et al., 1963). A synergistic anion such as carbonate (or bicarbonate) is essential for metal ion coordination (Bates & Schlabach, 1975).

A variety of ions including di- (Zweier, 1978; Aisen et al., 1969) and trivalent (Harris et al., 1974, 1975) transition metals, trivalent lanthanides (Luk, 1971; Meares & Ledbetter, 1977; Teuwissen et al., 1972), and tetravalent actinides (Harris et al., 1981) have been employed to help characterize the transferrin binding site. Luk (1971) has presented data for lanthanide–transferrin interactions which suggest that the two binding sites have different size restrictions for binding large metal ions.

Difference ultraviolet spectroscopy has been widely used to evaluate metal binding to transferrin (Gelb & Harris, 1980; Luk, 1971; Tan & Woodworth, 1969; Teuwissen et al., 1972). Metal complexation to the phenolic oxygen of a tyrosine residue perturbs the π to π^* transitions of the aromatic ring. The resulting absorbance changes are easily observed in the difference spectrum of the metalloprotein vs. the apoprotein since the absorbance for all nonbonding groups in the protein are blanked out of the spectrum. However, in order to determine the number of tyrosines bound to the metal ion, it is necessary to know the change in extinction coefficient $(\Delta\epsilon)$[2] for a single coordinated phenolate group vs. a protonated phenol. In previous studies, the assumed value of $\Delta\epsilon$ for coordination of a tyrosine anion to any metal ion has been

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* Address correspondence to this author at the Department of Chemistry, University of California.

‡ Present address: Laboratory for Energy-Related Health Research, University of California, Davis, CA 95616.

§ Present address: Department of Chemistry, University of Vermont, Burlington, VT 05405.

[1] By siderophilin we mean to describe the class of iron transport proteins, which includes the specific serum protein, transferrin.

[2] $\Delta\epsilon$ values for complexation of metal ions by transferrin and EHPG are based on the reaction $M^{n+} + HOPh \rightarrow [M^{n+}OPh]^{n-1} + H^+$. The $\Delta\epsilon$ for deprotonation of the phenolate ligand is $OH^- + HOPh \rightarrow {}^-OPh$.

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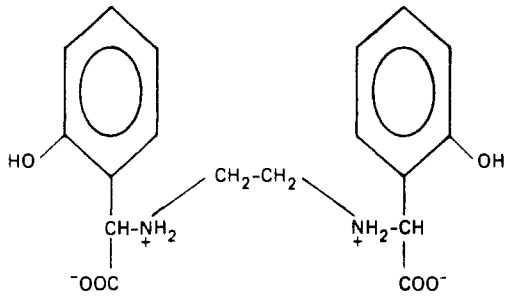

FIGURE 1: Molecular structure of EHPG.

10000 M^{-1} cm^{-1} [the $\Delta\epsilon$ for deprotonation of *N*-acetyltyrosine (Gelb & Harris, 1980; Luk, 1971; Tan & Woodworth, 1969; Aasa et al., 1963)]. Not only does this practice equate metal ion coordination with deprotonation but it also does not allow for any variation in $\Delta\epsilon$ for coordination of different metal ions. Thus, the large differences in total $\Delta\epsilon$ for transferrin complexes of di- and trivalent metal ions have been interpreted as an indication that three tyrosines are bound to trivalent ions whereas only two tyrosines are bound to divalent ions (Gelb & Harris, 1980).

We have now experimentally determined $\Delta\epsilon$ values for the binding of phenolate groups to a variety of metal ions by using ethylenebis[(*o*-hydroxyphenyl)glycine] (EHPG)[3] (Figure 1). This ligand forms very stable 1:1 complexes in which both phenolate groups are coordinated (Gaber et al., 1974; Anderegg & L'Eplattenier, 1964; Frost et al., 1958; Salama & Richardson, 1980). The results presented herein show that the $\Delta\epsilon$/phenol for metal coordination is not constant but varies substantially with the metal ion. The variations in intensities of the difference spectra of transferrin complexes of di- and trivalent metal ions are ascribed to variations in the intrinsic $\Delta\epsilon$ values rather than to any variation in the number of coordinated tyrosyl groups.

The results indicate that two tyrosines are coordinated per metal ion in the transferrin complexes of all the d-block transition metals and the lanthanides. Tan & Woodworth (1969) have previously suggested (based on difference UV spectra) that in transferrin two tyrosines coordinate per ferric ion. In addition, Gaber et al. (1974) have measured the resonance Raman spectra of $[Fe(EHPG)]^-$ and diferric transferrin and also suggested that two tyrosines are bound to iron. However, the data reported here are the first which suggest that the binding of two tyrosines per metal is a *general* feature of almost all metallotransferrin complexes.

Materials and Methods

Ethylenebis[(*o*-hydroxyphenyl)glycine] was obtained from Sigma Chemical Co. and was purified by washing with acetone under inert (N_2) atmosphere. The washed solid was dried in vacuo and stored under nitrogen. No attempt was made to separate the meso or DL isomers of EHPG before titration. All stock solutions of EHPG were prepared immediately prior to the difference titration.

Metal Stock Solutions. Stock solutions of $TbCl_3$, $ErCl_3$, and $HoCl_3$ (ROC/RIC), $ZnSO_4 \cdot 7H_2O$ (Mallinckrodt), $CuCl_2$ (Brothers), and $2CdSO_4 \cdot 5H_2O$ (Aldrich) were prepared in unbuffered distilled H_2O. Hydrochloric acid was added to $FeCl_3$, $VOSO_4$, and $GaCl_3$ solutions to prevent hydrolysis.

Difference Spectra. Ultraviolet difference spectra were recorded on a Cary 118 spectrophotometer between 215 and 325 nm. For titrations of the lanthanides and divalent metal ions, EHPG was dissolved in 0.1 M borate buffer (pH 9.8). Stock solutions of EHPG for Fe^{3+} were buffered at pH 5.5 with 0.1 M Mes. Ten-microliter aliquots of metal were added to the sample cuvette, and 10-μL of H_2O was added to the reference cell with a 2.0-mL Gilmont micrometer buret. The initial sample volume in each case was 3 mL, and a titration typically required 150 μL for completion. Thus, a 5% change in volume occurred in both cuvettes during the course of the titration. The lanthanides displayed slow reaction kinetics due to hydrolysis of the metal; therefore, spectra were recorded at 30-min intervals. All other metal complexation reactions were essentially instantaneous. The lanthanides and divalent metals were titrated at pH 9.8 to assure that complex formation was complete. Since the first phenolic pK_a of EHPG is 10.24 (Anderegg & L'Eplattenier, 1964), the $\Delta\epsilon$ values determined for these metals have been corrected for the deprotonation equilibrium in this region by using a value of $\Delta\epsilon_{prot}$ = 9600 for the deprotonation of EHPG. These corrections were never greater than 20% of the observed spectral value. All pH measurements were made on a Beckman pH-102 Metrohm pH meter using a Sigma combination pH electrode. For the vanadyl solutions, strict anaerobic conditions were maintained.

Transferrin Titrations. Human Serum Transferrin was purchased from Sigma and purified by elution from a freshly prepared Sephadex G-25 column with first 0.1 M $NaClO_4$ and 0.05 M Tris, pH 7.5, and then 0.05 M Tris, pH 8.6. The concentration of apotransferrin (M_r 77 000) was determined from the UV absorbance at 278 nm by using $\epsilon = 9.23 \times 10^4$ M^{-1} cm^{-1} (Chasteen, 1977).

Holmium was administered as the $[Ho(NTA)_2]^{3-}$ complex following the procedure described above. Just before the addition of metal, $KHCO_3$ was added (5 mM final total concentration) to give a carbonate-saturated solution. It appeared that NTA competed with transferrin for Ho^{3+}; therefore, in order to assure complete saturation of transferrin, the last addition of Ho^{3+} was as the chloride. The final solution had only a 5% excess of NTA over calculated free metal. Since NTA competed with transferrin for both Er^{3+} and Eu^{3+}, the metals were administered as the chlorides. Gallium transferrin titrations were carried out in the absence of NTA, as in Ford-Hutchinson & Perkins (1970), and with a concentration of NTA in the transferrin solution to form a bis complex with 25% of the total theoretical Ga^{3+} needed to saturate the protein. The NTA was added to avoid the possible hydrolysis of Ga^{3+}. At the above concentration NTA had no effect on the initial slope in a plot of $\Delta\epsilon$ vs. r ([metal]/[transferrin]).

Deprotonation Difference Spectra. A standard solution of EHPG was titrated into 0.1 M KOH. The $\Delta\epsilon$ was then calculated from the slope of a plot of ΔA vs. added ligand.

Results

EHPG Difference Spectra. Typical spectra for metal ion complexation by EHPG are shown in Figure 2. Two maxima are observed at 242 and 293 nm as well as a minimum (negative absorbance) at 269 nm. The copper(II), iron(III), and VO^{2+} spectra were anomalous in that the negative intensity minimum was not observed.

At neutral or acidic pH, the phenolic oxygens of the free ligand are completely protonated. Metal coordination displaces these protons, so the absorption spectrum reflects the differences in absorptivities between the metal complex and the protonated form of the ligand. When spectra are recorded

[3] Abbreviations used: EHPG, ethylenebis[(*o*-hydroxyphenyl)glycine]; NTA, nitrilotriacetic acid; Mes, 2-(*N*-morphilino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane hydrochloride; $FeEDTA^-$, ferric ethylenediaminetetraacetic acid anion; HBED, *N*,*N*'-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid.

Table I: Values of $\Delta\epsilon$ for Binding of Metal Ions to EHPG and Transferrin and an Estimation of the Number of Tyrosyl Residues Involved in Metal Coordination to Transferrin

metal	ionic radius[a]	$\Delta\epsilon^{EHPG}$ ($\times 10^{-3}$)[b]	$\Delta\epsilon^{Tr}$ ($\times 10^{-3}$)	metals bound to transferrin	no. of Tyr residues
Cu^{2+}	0.73^{VI}, 0.57^{IV}	5.2 ± 0.3	21.0[c] (19.9,[d] 22.0[d])	2	4.0 ± 0.4
Cd^{2+}	0.95^{VI}	5.1 ± 0.3	22.4[d]	2	4.4 ± 0.4
Zn^{2+}	0.74^{VI}, 0.60^{IV}	6.7 ± 0.3	24.5[c] (24.9,[d] 24.0[e])	2	3.7 ± 0.4
VO^{2+}		8.3 ± 0.3	40.0[b]	2	4.8 ± 0.5
Eu^{3+}	0.947^{VI}, 1.066^{VIII}	7.6 ± 0.3	31.6[e]	2	4.2 ± 0.5
			32.2		4.3 ± 0.5
Er^{3+}	0.890^{VI}, 1.004^{VIII}	7.5 ± 0.3	37.0[e]	2	4.9 ± 0.5
			32.5[b]		4.3 ± 0.4
Tb^{3+}	0.923^{VI}, 1.040^{VIII}	7.9 ± 0.3	37.5[e]	2	4.8 ± 0.5
			35.4[f]		4.5 ± 0.5
Ho^{3+}	0.901^{VI}, 1.015^{VIII}	7.8 ± 0.3	37.0[e]	2	4.7 ± 0.5
			33.0[b]		4.2 ± 0.4
Ga^{2+}	0.62^{VI}	7.4 ± 0.3	36.0[g]	2	4.9 ± 0.5
			32.0[h]		4.3 ± 0.5
			35.3[b]		4.7 ± 0.5
Fe^{3+}	0.645^{VI}	8.7 ± 0.3	36.3[d]	2	4.2 ± 0.4
Th^{4+}	0.94^{VI}, 1.05^{VIII}	8.7 ± 0.3	25.0[i]	2	2.9 ± 0.3
Nd^{3+}	0.983^{VI}, 1.109^{VIII}	7.7[j]	17.0[e]	1	2.2 ± 0.3[j]
Pr^{3+}	0.99^{VI}, 1.126^{VIII}	7.7[j]	14.0[e]	1	1.8 ± 0.3[j]
Pu^{4+}	0.86^{VI}, 0.96^{VIII}				

[a] Values from Shannon (1976). Roman numerals indicate the metal ion coordination number for each ionic radius. [b] Determined in this work. [c] Average of two values from different laboratories. [d] Tan & Woodworth (1969). [e] Luk (1971). [f] Teuwissen et al. (1972). [g] Gelb & Harris (1980), transferrin. [h] Gelb & Harris (1980), ovotransferrin. [i] Harris et al. (1981). [j] Estimated using the average $\Delta\epsilon$ for Ln^{3+}-EHPG complexation (7.7×10^3).

below pH 9, the $\Delta\epsilon$ values are calculated by dividing the absorbance by the total ligand concentration. The $\Delta\epsilon$ is then plotted vs. r, the ratio of $[M]_{tot}/[L]_{tot}$.

When measurements are made in solutions at pH >9, it becomes necessary to correct the observed $\Delta\epsilon$ for the equilibrium concentration of free deprotonated ligand. This correction is based on the known ligand protonation constants and the $\Delta\epsilon_{prot}$ between deprotonated and protonated forms of the ligand. We can treat EHPG as a monoprotic system for this correction since the ligand protonation equilibria are sufficiently separated from each other. The value of $\Delta\epsilon_{prot}$ was measured by titrating ligand into 0.10 M KOH and using an equimolar ligand solution in distilled water as a blank. The value of $\Delta\epsilon_{prot} = 9600\ M^{-1}\ cm^{-1}$ is very close to the value of $10\,000\ M^{-1}\ cm^{-1}$ previously reported for the deprotonation of *N*-acetyltyrosine.

The fraction of deprotonated free ligand (α) is calculated from the first phenolic pK_a of 10.24.

$$\alpha = \frac{1}{1 + ([H^+]/K_a)} \quad (1)$$

This ratio is fixed by the pH and is independent of the metal ion concentration. Thus the absorbance produced by the addition of metal ion is the sum of two components representing the differences in absorptivity between the metal complex and both the protonated and deprotonated form of the ligand. The apparent $\Delta\epsilon$ is given by

$$\Delta\epsilon_{app} = (1 - \alpha)\Delta\epsilon + \alpha(\Delta\epsilon') \quad (2)$$

where $\Delta\epsilon$ and $\Delta\epsilon'$ represent the values for the metal complex vs. the protonated and deprotonated form of the ligand, respectively. The $\Delta\epsilon'$ value is simple ($\Delta\epsilon - \Delta\epsilon_{prot}$) so that eq 2 can be rearranged to

$$\Delta\epsilon = \Delta\epsilon_{app} + \alpha\Delta\epsilon_{prot} \quad (3)$$

The plots of $\Delta\epsilon$ vs. r all have sharp breaks near $r = 1$, confirming the 1:1 stoichiometry of the metal–EHPG complex (Figure 3). However, the magnitude of $\Delta\epsilon$ at $r = 1$ varies considerably for different metals. The pH for each metal system was chosen on the basis of previously reported potentiometric titration data, such that both the EHPG phenolic groups would be completely coordinated to the metal ion. Thus the variations in $\Delta\epsilon$ do not reflect any changes in the degree of coordination of the phenols but rather intrinsic differences in the $\Delta\epsilon$ for phenol coordination to various metals. Since the measured $\Delta\epsilon$ represents coordination of two phenolic groups, half this value gives $\Delta\epsilon$ phenol for each of the metal ions. These values are listed in Table I.

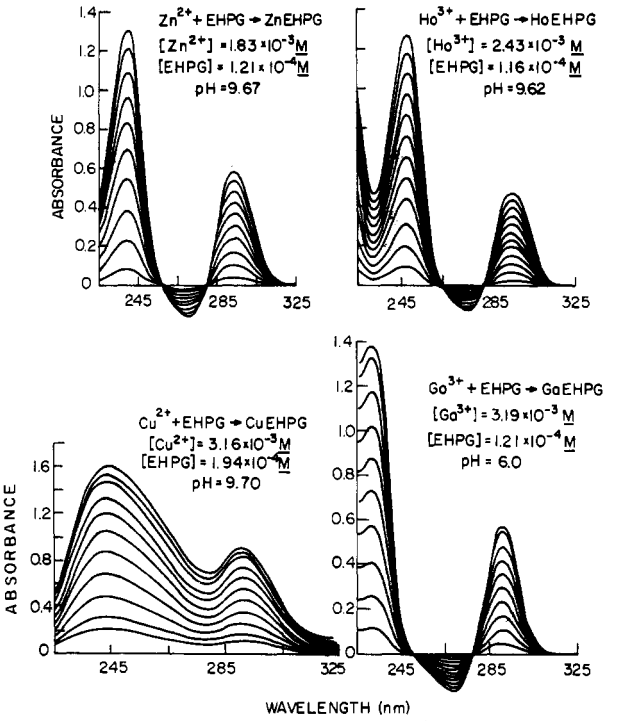

FIGURE 2: Difference ultraviolet spectra of metal–EHPG titrations. (a) Zn^{2+}, $[Zn^{2+}] = 1.80 \times 10^{-3}$ M, $[EHPG] = 1.16 \times 10^{-4}$ M, pH 9.67; (b) Cu^{2+}, $[Cu^{2+}] = 3.16 \times 10^{-3}$ M, $[EHPG] = 1.94 \times 10^{-4}$ M, pH 9.70; (c) Ho^{3+}, $[Ho^{3+}] = 2.43 \times 10^{-3}$ M, $[EHPG] = 1.16 \times 10^{-4}$ M, pH 9.62; (d) Ga^{3+}, $[Ga^{3+}] = 3.19 \times 10^{-3}$ M, $[EHPG] = 1.10 \times 10^{-4}$ M, pH 6.0.

Transferrin Difference Spectra. For a number of metal ions, spectrophotometric titrations were performed by using

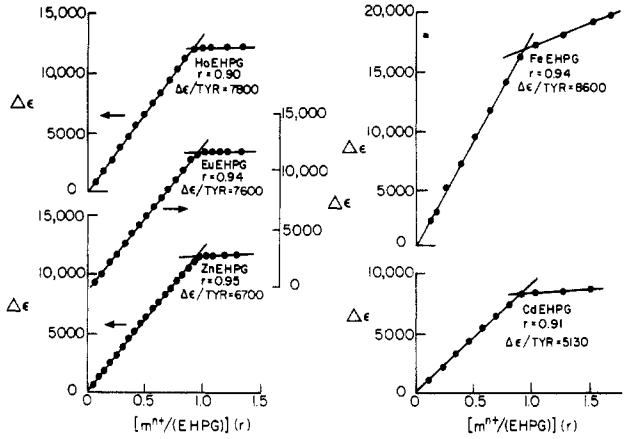

FIGURE 3: Titration of EHPG with Ho^{3+}, Eu^{3+}, Zn^{2+}, Fe^{3+}, and Cd^{2+}. Ho^{3+}, $[Ho^{3+}] = 2.43 \times 10^{-3}$ M, [EHPG] = 1.16×10^{-4} M, pH 9.62; Eu^{3+}, $[Eu^{3+}] = 1.03 \times 10^{-3}$ M, [EHPG] = 1.94×10^{-4} M, pH 9.70; Zn^{2+}, $[Zn^{2+}] = 1.80 \times 10^{-3}$ M, [EHPG] = 1.16×10^{-4} M, pH 9.67; Fe^{3+}, $[Fe^{3+}] = 1.51 \times 10^{-3}$ M, [EHPG] = 1.94×10^{-4} M, pH 5.5; Cd^{2+}, $[Cd^{2+}] = 1.91 \times 10^{-3}$ M, [EHPG] = 1.16×10^{-4} M, pH 9.8. (The observed $\Delta\epsilon_{242}$ is not corrected for EHPG protonation equilibria which occurred for titrations at high pH.)

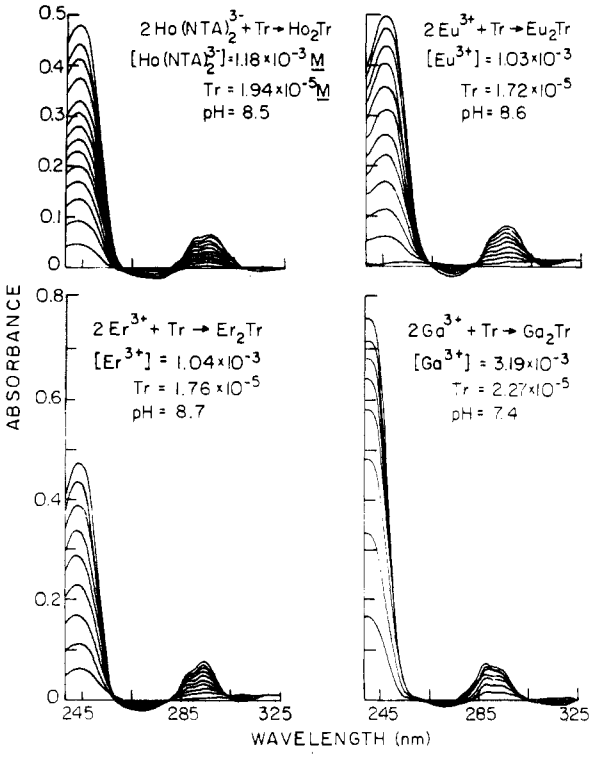

FIGURE 4: Difference ultraviolet spectra for the reaction of $[Ho(NTA)_2]^{3-}$, $ErCl_3$, $EuCl_3$, and $Ga(NTA)_2^{3-}$ with apotransferrin in Tris buffer. $[[Ho(NTA)_2]^{3-}] = 1.18 \times 10^{-3}$ M, [transferrin] = 1.94×10^{-5} M, pH 8.5; $[ErCl_3] = 1.04 \times 10^{-3}$ M, [transferrin] = 1.76×10^{-5} M, pH 8.7; $[EuCl_3] = 1.03 \times 10^{-3}$ M, [transferrin] = 1.72×10^{-5} M, pH 8.6; $[Ga(NTA)_2^{3-}] = 3.19 \times 10^{-3}$ M, [transferrin] = 2.27×10^{-5} M, pH 7.4.

transferrin instead of EHPG. Solutions of $[Ho(NTA)_2]^{3-}$, $ErCl_3$, $EuCl_3$, $Ga(NTA)_2$, and $Ga(NO_3)_3$ were used as the titrants. The absorption spectra, shown in Figure 4, are obviously quite similar to the EHPG difference spectra. The plot of $\Delta\epsilon$ vs. r (Figure 5) for the Ho^{3+}, Er^{3+}, and Eu^{3+} plus transferrin systems have a sharp break at $r = 2$, indicating the binding of two metal ions to each transferrin molecule. We observed that a plot of $\Delta\epsilon$ vs. r for complexation of Ga^{3+} by transferrin did not exhibit a sharp break at $r = 2$. Subsequently (W. R. Harris, V. L. Pecoraro, and K. N. Raymond, unpublished results), we have shown that NTA can effectively

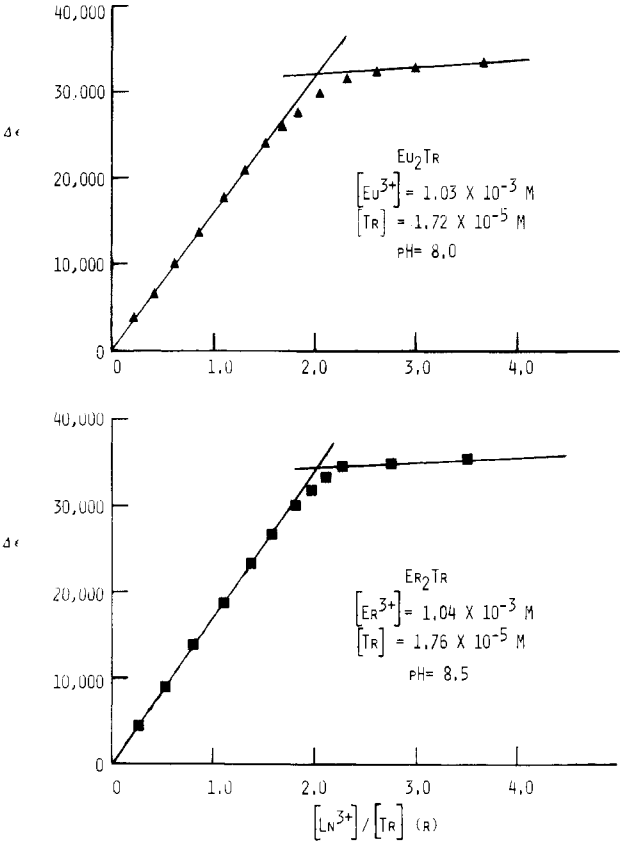

FIGURE 5: Titration of transferrin with $ErCl_3$ and $EuCl_3$. The abscissa is the ratio of [metal]/[transferrin], and the ordinate is the observed $\Delta\epsilon$. Eu^{3+}, $[Eu^{3+}] = 1.03 \times 10^{-3}$, $[Tr] = 1.72 \times 10^{-5}$, pH 8.6; Er^{3+}, $[Er^{3+}] = 1.04 \times 10^{-3}$, $[Tr] = 1.76 \times 10^{-5}$, pH 8.7.

compete with transferrin for Ga^{3+}. Thus our data indicate that complexation of Ga^{3+} by transferrin is weaker than the corresponding ferric complex. Therefore, the $\Delta\epsilon$ for Ga^{3+}-transferrin was determined by extrapolating the initial slope, the only region where complex formation is complete, to $r = 2$. This value is in good agreement with the previously reported $\Delta\epsilon$ (Gelb & Harris, 1980). The total $\Delta\epsilon$ for the binding of two metal ions are listed in Table I.

Discussion

In previous difference UV studies on transferrin complexes, it has always been assumed that the $\Delta\epsilon$/phenol for any metal–tyrosine complex vs. protonated tyrosine was equal to the experimentally determined $\Delta\epsilon$ of 10000 M^{-1} cm^{-1} for the deprotonated vs. the protonated form of *N*-acetyltyrosine (Gelb & Harris, 1980; Luk, 1971; Tan & Woodworth, 1969). Such an assumption implies that the influence of the metal on the ultraviolet spectrum is limited to its ability to displace a proton and that perturbations of the relative π to π^* orbital energies of the phenolic ring due to metal–oxygen binding are negligible. Rather than make such an assumption, we have determined the actual magnitude of $\Delta\epsilon$ for the coordination of a series of metal ions to phenolic groups. These values are based on the difference UV spectra of metallo–EHPG complexes. The results clearly indicate *substantial* variations in $\Delta\epsilon$ for different metals, ranging from a low of 5100 M^{-1} cm^{-1} for Cd^{2+} to a maximum of 8700 M^{-1} cm^{-1} for Th^{4+} and Fe^{3+}.

The $\Delta\epsilon$/phenol values fall into two categories: divalent metal ions, $\Delta\epsilon$ = 5100–6700 M^{-1} cm^{-1}, and trivalent metals and tetravalent actinides, $\Delta\epsilon$ = 7400–8700 M^{-1} cm^{-1}.

There are two factors which govern the magnitude of $\Delta\epsilon$ for metal coordination. The first is the change in intensity of the π to π^* band due to metal ion (rather than proton)

Table II: Comparison of the Physical Properties of FeEHPG and Diferric Transferrin

	FeEHPG	Fe_2Tr
visible spectra	λ_{max}[a] = 461 nm	λ_{max} = 466 nm
	ϵ[a] = 2758 M^{-1} cm^{-1}	ϵ[b] = 2500 M^{-1} cm^{-1}
	water, pH 11[c]	water, pH 7.4
redox potential	-642 mV vs. SCE[d]	[e]
EPR parameters	tensor, g'[f]	tensor, g'[g]
	x = 4.30	x = 4.27
	y = 4.24	y = 4.08
	z = 4.38	z = 4.39
Mössbauer parameters	δ_{Fe} = 0.37 mm/s[h]	δ_{Fe} = 0.38 mm/s[h]
	ΔEq = 0.77 mm/s[h]	ΔEq = 0.75 mm/s[h]
resonance Raman spectra	FeEHPG and diferric transferrin are very similar[i]	

[a] M. G. Patch, K. S. Simolo, and C. J. Carrano, unpublished results. [b] Epsilon value is per mole of iron. [c] At pH 11 in aqueous solution, FeEHPG forms a six-coordinate complex in which two phenol oxygens, a carboxylate oxygen, and two amine nitrogens are derived from EHPG, with the sixth coordination site being filled by hydroxide. For more details, see reference in *a* above. [d] At pH 9 and above. Schrøder (1964). [e] The redox potential for differric transferrin in unknown; however, the very negative potential for FeEHPG is consistent with the observation that the ferrous transferrin species is very weak if, indeed, it exists. [f] g' values are for the *rac*-FeEHPG complex in glycerine/water (5:1 v/v). Details are given in the reference in *a* above. [g] Aasa (1972). [h] An unspecified mixture of *meso*- and *rac*-FeEHPG. Data obtained from Ainscough et al. (1980). [i] An unspecified mixture of *meso*- and *rac*-FeEHPG. Data obtained from Gaber et al. (1974).

binding. Second, the perturbation of the relative π and π^* orbital energies is altered via metal complexation, causing a shift in λ_{max} for the peak. Although it is very difficult to present a theoretical basis for the observed trend in $\Delta\epsilon$, it appears that the magnitude of $\Delta\epsilon$ roughly correlates with the charge to radius ratio of the metal ion. The major exception to this trend is GaEHPG. Since Ga^{3+} and Fe^{3+} have very similar charge to radius ratios, a value closer to 8700 M^{-1} cm^{-1} might be expected; however, we have reproduced the 7400 M^{-1} cm^{-1} value many times. We are presently investigating the effect on $\Delta\epsilon$ for coordination of other polyaminocarboxylate ligands, such as $(HBED)^3$ with Ga(III), in an effort to understand the apparently low $\Delta\epsilon$ for GaEHPG.

The difference spectra of EHPG and transferrin complexes are quite similar, even though EHPG contains *o*-phenolic groups while the tyrosine residues of transferrin contain *p*-phenols. In addition, the data summarized in Table II show marked similarities between the FeEHPG model and diferric transferrin. Furthermore, the $\Delta\epsilon^{242}_{prot}$ values for EHPG and *N*-acetyltyrosine are very close. Therefore, we feel that the $\Delta\epsilon$/phenol values determined for the EHPG complexes are good estimates of the $\Delta\epsilon$/tyrosine for the corresponding transferrin complexes. The total $\Delta\epsilon$ values have been reported for several dimetallotransferrin complexes. Therefore, we are now able to calculate the number of coordinated tyrosines simply as

$$n = \frac{\text{total } \Delta\epsilon}{\Delta\epsilon/\text{phenol}} \quad (4)$$

The value of n for a series of metals is shown in Table I. The values for the d-block transition metals are all very close to 4.0, strongly suggesting the coordination of two tyrosines per metal ion at each site. The $\Delta\epsilon$ for the Ga_2–transferrin complex is 36 000, which is quite similar to that of diferric transferrin. The low $\Delta\epsilon^{EHPG}$ value (see above) gives a high n for gallium complexation by transferrin. However, we believe that we can still distinguish between four and six coordinated tyrosines and

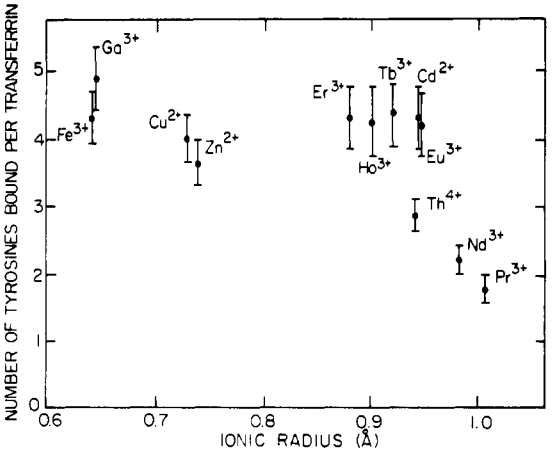

FIGURE 6: Ability of transferrin to engage in tyrosyl coordination of metal ions as a function of ionic radius.

that in Ga_2-transferrin only four phenols are bound to the metal ions.

The n values for Er^{3+}, Tb^{3+}, and Ho^{3+}, based on Luk's (1971) transferrin results (Rogers et al., 1977), appear to be high. (Similarly, the value of $\Delta\epsilon$ for VO^{2+}–transferrin, 40 000, appears to be high.) A separate report for Tb_2-transferrin gave a somewhat lower value of 17 250 per Tb^{3+} (Teuwissen et al., 1972). During our experiments on lanthanide–EHPG systems, we observed very slow equilibration which we attributed to hydrolysis of the metal ions. The titration curves presented by Luk (1971) do not give sharp end points (indicating weak or competitive binding of the administered metal ion); moreover, Luk's (1971) date extrapolate to unreasonable r values (in many cases, indicating greater than two metals bound). For these reasons, we repeated transferrin titrations using $Ho(NTA)_2$, $ErCl_3$, and $EuCl_3$ and have obtained lower values for $\Delta\epsilon$. All the titrations (Figure 5) gave sharp end points which extrapolated back to r = 2. The values of n based on these data and those reported by Teuwissen et al. (1972) are around 4.2–4.3. It appears that there are in fact at most four coordinated tyrosines in all the lanthanide–transferrin complexes. This conclusion is supported by the $\Delta\epsilon$ values for the Nd^{3+} and Pr^{3+} transferrin complexes. Since these are among the largest of the lanthanide ions, they should have a lower tendency to hydrolyze. Luk (1971) has shown that only one Nd^{3+} or Pr^{3+} binds to transferrin, to give a $\Delta\epsilon \simeq 15\,000$. On the basis of an average lanthanide $\Delta\epsilon$/phenol of 7700, this corresponds to two tyrosines per bound metal ion.

The thorium transferrin system is unique, with a total of three tyrosines bound to two metal ions. It has been shown that in the stronger of the two transferrin binding sites, two tyrosines are coordinated to thorium but that in the weaker binding site, only a single tyrosine is bound (Harris et al., 1981).

The values of n listed in Table I are plotted vs. the metal ionic radii (Shannon, 1976) in Figure 6. It is obvious that one of the transferrin binding sites cannot accommodate very large metal ions. Unfortunately, it is not possible to determine exactly this critical size restriction from these data since the ionic radius of a metal ion varies with its coordination number and the coordination numbers of the metal ions in their transferrin complexes are unknown. A semiquantitative evaluation suggests that metal ions 0.02 Å smaller than Eu^{3+} are bound by the maximum number of tyrosines available whereas larger metal ions are incompletely coordinated. This observation suggests that the use of Th^{4+} as a model for Pu^{4+} biochemistry may be inadequate since the relative ionic radius

of Pu^{4+} is 0.08 Å less than the borderline Th^{4+} (Salama & Richardson, 1980). This size difference decreases the ability of a tyrosine to bind Th^{4+} in one binding site. The difference in the organ distribution (Durbin, 1960; Singh et al., 1979) for Pu^{4+} and Th^{4+} may also be a manifestation of this phenomenon.

The variations in the transferrin $\Delta\epsilon$ values often have been ascribed to the coordination of either two or three tyrosines per metal ion. However, the EHPG results discussed above show that these variations are due to changes in $\Delta\epsilon$/phenol for different metal ions and that there seems to be *two tyrosines* coordinated to all metal ions except for one of the two thorium ions. These results must now be reconciled with proton release studies which show that *three protons* are displaced by ferric ion while *two protons* are displaced by cupric ion.

Two of the three protons displaced by ferric ion must come from tyrosine residues. The proton release studies are conducted above pH 7, so histidyl groups ($pK_a \simeq 6.0$) are unlikely sources for additional protons. Gelb & Harris (1980) have shown that three protons are released even when oxalate is used as the only synergistic anion, so the third proton cannot come from bicarbonate. However, NMR data on diferric transferrin indicate that there is a water molecule directly coordinated to the ferric ion (Koenig & Schillinger, 1969). The tendency of ferric complexes to hydrolyze at neutral pH is well established, particularly when there is a water molecule within the inner coordination sphere. Moreover, this tendency for ferric ion hydrolysis is so strong that $(FeEDTA)^-$ forms a seven-coordinate aquo species which undergoes hydrolysis at pH 8 (Gustafson & Martell, 1963). This suggests that the third proton released by complexation of ferric ion is due to the deprotonation of this coordinated water and that the iron is actually bound to transferrin as a hydroxo species. Although the previously reported NMR data do not distinguish bound water from hydroxide, our model is supported by the relaxation rates (Koenig & Schillinger, 1969) which are pH independent between 6.5 and 9.4.[4] This suggests that the hydroxide ion is bound over the entire range of hydrogen ion concentration.

The ability of bicarbonate to act as the synergistic anion in the ferric transferrin complex has been demonstrated to be much greater than NTA (Schlabach & Bates, 1975). It has been assumed here that bicarbonate acts as the synergistic anion in all the metallotransferrin complexes since this has invariably been proven to be the case unless special precautions to exclude HCO_3^- are taken. Thus, it is possible that the complex actually binds CO_3^{2-} and that the two extra protons released upon metal complexation are due to the deprotonation of HCO_3^-. On the basis of anion binding studies for metallotransferrin complexation as well as experiments substituting oxalate for bicarbonate as the synergistic anion (Gelb & Harris, 1980), we do not favor this conclusion; however, our data would not contradict such a proposal.

Thorium(IV) also has a strong tendency to hydrolyze (Mesmer & Baes, 1976), and we have previously shown that a total of five protons are displaced upon binding of two thorium ions (Harris et al., 1981). Three of the five protons are displaced from coordinated tyrosyl groups, but the remaining two are attributed to the deprotonation of coordinated water molecules by this strongly polarizing Lewis acid. Conversely, each copper(II) displaces only two protons from transferrin, presumably due to the coordination of two tyrosines. The absence of any additional protons is consistent with the much smaller hydrolytic tendency of Cu^{2+} compared to Fe^{3+} or Th^{4+}.

On the basis of these data, we wish to propose a new model for the iron(III) transferrin binding site, in which the iron is coordinated to two tyrosines, two histidines, hydroxide ion, and bicarbonate. Such a model is consistent with the UV difference spectra, chemical modification studies (Rogers et al., 1977; Zschocke et al., 1972; Buttkus et al., 1965; Ford-Hutchinson & Perkins, 1970), and proton release data (Ford-Hutchinson & Perkins, 1970; Gelb & Harris, 1980; Aasa et al., 1963). Since the actual measurement of the $\Delta\epsilon$ values for each metal shows these to vary widely, there now appears to be much more consistency in the binding of various metal ions to transferrin; it is no longer necessary to propose changes in the protein donor groups to account for variations in $\Delta\epsilon$ and the number of protons released by various metals. Instead, these variations can be explained in terms of the chemical properties of the metal ions themselves.

Summary

Monitoring the difference ultraviolet spectra for metal ion complexation to EHPG gives a more accurate $\Delta\epsilon_{242}$ value than the previously used standard for metal–tyrosyl coordination in proteins. The new model demonstrates a significant difference in the magnitude of $\Delta\epsilon_{242}$ for various metals investigated, suggesting that previous estimations of the number of tyrosines ligated to a metal may be incorrect. For divalent cations, two tyrosyl moieties are predicted to be coordinated to the metal at each site. This is in agreement with proton release studies which show two H^+ ions released per Cu^{2+} or Zn^{2+} bound to transferrin. Moreover, it appears that two tyrosine residues are coordinated to all metals which are smaller than a critical ionic radius.

Lanthanide ions offer a convenient sequence of metals which are suitable probes of this size dependence due to a monotonic decrease in ionic radius across the series and a uniform charge. Since in the transferrin complex, the coordination number of the Ln^{3+} ions are unknown, it is impossible by these methods to place an absolute restriction on the size of the metal site. However, it appears that the radius of Eu^{3+} is a relative maximum limit at which two tyrosines can be coordinated to the metal.

In a separate paper (Harris et al., 1981), we describe the complexation of Th^{4+} by transferrin in detail. The ionic radius of this ion lies on the borderline between complete and incomplete tyrosyl coordination, and it is observed that only three tyrosines are bound to the metals in Th_2-transferrin. One tyrosine is coordinated to the thorium at pH 7.2 in the N-terminal site whereas two tyrosines were bound at the C-terminal site. We suggest that (a) this is direct evidence for the inequivalence of the two transferrin binding sites and (b) the N-terminal site is the center exhibiting the size-dependent tyrosyl ligation at physiological pH.

Thorium has been used as a model for the biochemistry of plutonium because of its similar charge, size, and low specific radioactivity. Our data indicate that the size discrepancy exhibited by these two metals (~0.08 Å) is sufficiently great to allow transferrin to distinguish Pu^{4+} and Th^{4+}. This may

[4] The degree to which the Koenig & Schillinger NMR data support or refute our proposed model has been raised by a referee. We note that the NMR data cannot alone distinguish between proton and water (or hydroxide) exchange. Furthermore, the observation that there is no pH dependence in the observed exchange does not rule out hydrolysis of the metal as we propose. Several mechanisms may be written which involve proton transfer in the exchange process but not in the rate-determining step. Indeed, exactly such a pH independence has been found for Co^{2+}-substituted carbonic anhydrase and a model compound *even though the metal is clearly present as the hydroxide complex at neutral pH* (Bertini et al., 1981).

explain why the distribution of Pu^{4+} in mammalian tissue is different from that of the Th^{4+} ion and this makes us question whether Th^{4+} is a good biological model for Pu^{4+}.

The magnitude of $\Delta\epsilon_{242}$ for FeEHPG complexation is 8700 cm^{-1} M^{-1}. On the basis of this value, four tyrosines are expected to be coordinated to the two irons in diferric transferrin. We have suggested a model for the coordination environment of the ferric ion which is consistent with proton release studies. In addition to the two tyrosines, there are probably two histidine residues, a bicarbonate, and a hydroxide which form a six-coordinate complex. Two of the three protons liberated upon complexation of the metal are released from the tyrosines, while the remaining proton originates from hydrolysis of the coordinated water molecule. A similar hydrolysis is observed for the complexation of Th^{4+} to transferrin.

Added in Proof

A recent ^{13}C NMR study (Zweier et al., 1981) has probed the difference in anion binding of the two Fe^{3+} binding sites of transferrin.

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