Gadolinium(III) appears to be a suitable paramagnetic probe for studying the macromolecular environment of metal ions by magnetic relaxation methods. Other members of the lanthanides series, with very short electron spin relaxation times (Reuben and Fiat, 1969) can be used to produce isotropic nuclear resonance shifts (Morallee *et al.*, 1970).

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Study of the Nature of the Metal-Binding Sites and Estimate of the Distance between the Metal-Binding Sites in Transferrin Using Trivalent Lanthanide Ions as Fluorescent Probes*

Chun Ka Luk

ABSTRACT: Trivalent lanthanide ions were used as fluorescent probes in the study of transferrin conformation. It was found that there are two specific binding sites per transferrin molecules for Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+}, and that there is only one specific binding site per transferrin molecule for Nd^{3+} and Pr^{3+}. The latter ions have larger ionic radii than the former. It was also shown that two tyrosyl residues are involved in each terbium binding site. Studies by fluorescence spectroscopy showed that terbium ion is bound to the phenolic oxygen of the tyrosyl residues. The small deuterium solvent effect on the Tb^{3+} fluorescence in the complex indicates that very few water molecules are bound to terbium.

From the lack of energy transfer between a Tb^{3+} and Fe^{3+} bound to the same protein, it is found that the distance between the two specific metal binding sites is equal to or greater than 43 Å.

Human serum transferrin is an iron-binding protein acting as an iron buffer and also as an iron carrier. Transferrin, whose molecular weight is 77,000 (Mann *et al.*, 1970), is probably a prolate ellipsoid of axial ratio 1:3 (Bezkorovainy and Rafelson, 1964). It possesses two iron-binding sites which are also capable of binding a number of other metal ions specifically and tightly. Much of the chemistry of transferrin was reviewed recently (Aisen, 1971). In summary, it has been found that the two binding sites are probably equivalent and greater than 9 Å apart; that four nitrogen ligands are available at each site; that a few tyrosyl residues are probably involved in metal binding; and that bicarbonate is involved in the iron binding.

In this paper, fluorescence techniques were used to study the nature of metal-binding sites and the separation of the two binding sites. Fluorescent probes were used and measurements on energy transfer were involved. The fluorescent probes used were trivalent lanthanide ions and ferric and cupric ions were used as quenchers.

Experimental Section

Purified human transferrin (iron free) was purchased from Behring Diagnostics, Inc. (Woodbury, N. Y.), and was used

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without further purification. $TbCl_3 \cdot 6H_2O$, $ErCl_3 \cdot 6H_2O$, $EuCl_3 \cdot 6H_2O$, $HoCl_3 \cdot 6H_2O$, $NdCl_3 \cdot 6H_2O$, and $PrCl_3 \cdot 6H_2O$ of highest purified grade available were purchased from Alfa Inorganics, Inc. (Beverly, Mass.), and were also used without further purification.

Absorption spectra were taken on Cary 14 spectrophotometer. Fluorescence spectra were taken on a fluorescence spectrophotometer Model MPF-2A sold by Perkin-Elmer Co. All fluorescence spectra were not corrected for the spectral response of the instrument. Fluorescence lifetimes were measured by using a Novatron-789A nanopulse lamp with Model 437 nanopulser manufactured by Xenon Corp. (Medford, Mass.) and an Amperex 56 DUVP photomultiplier placed 90° to the excitation source and after an emission monochromator. The signal from the photomultiplier is fed into a Tektronix 1A7A amplifier with 1-MHz bandwidth. The output of the amplifier is then fed into a waveform eductor (Princeton Applied Research) to increase signal-to-noise ratio. The results were read out on a X–Y recorder and analyzed by the method of least squares.

A filter (Corning 7-54) placed between excitation source and sample was also used to eliminate scattering light from the excitation lamp. Since the excitation lamp has a pulse width of 20 nsec, the time resolution of the lamp pulse and the emission decay curves is excellent.

Glass-distilled water was used throughout the experiment. The concentration of the transferrin was about 10^{-5} M in Tris-HCl buffer (ionic strength 0.01, pH 8.5) and 0.005 M $NaHCO_3$ and was estimated spectrophotometrically with the use of extinction coefficient, $\epsilon_{278m\mu} = 9.23 \times 10^4 M^{-1} cm^{-1}$.

Titrations of transferrin solution with various metal ions were done as follows: to each of the aliquot portion (5 ml) of the transferrin solution ($\sim 10^{-5}$ M), different amounts of the concentrated trivalent lanthanide ion solution (Stork solution, $2.0 \pm 0.2 \times 10^{-3}$ M) were added with a manostat digit pipet. The total change in the volume of the solutions never exceeds 1% after titration. The ultraviolet difference absorption spectrum was then taken at least 48 hr after the solutions were prepared. The ultraviolet difference absorption spectrum appeared to be the same whether measurements were made 0.5 or 48 hr after the solutions were prepared. In case of Tb^{3+}, the titrated solutions were also studied spectrofluorometrically.

To determine the relative binding strength of the rare earth ions, an equal amount of the europium, holmium, erbium, neodymium, and praseodymium ions equivalent to the total terbium ion content was added to each of the aliquot portions of Tb^{3+}–transferrin solution in which the metal-binding sites of the transferrin were saturated with Tb^{3+} (Tris-HCl buffer, pH 8.5, $\mu = 0.01$, and 0.005 M $NaHCO_3$). The fluorescence intensity of the terbium ion was monitored and compared. The relative strength of binding of the rare earth ions to transferrin was then deduced from their ability to displace terbium ions from the transferrin molecules, since the fluorescence intensity of unbound terbium ion is negligible.

Determination of the distance between the binding sites was done by adding Eu^{3+}, Ho^{3+}, Er^{3+}, Cu^{2+}, or Fe^{3+} (amount equivalent to total Tb^{3+} content of a solution) to a Tb^{3+}–transferrin solution where the metal-binding sites of the transferrin were just slightly less than half-saturated. The Tb^{3+} fluorescence intensity excited at 295 mμ and its fluorescence lifetime was then measured. Possible quenching of Tb^{3+} fluorescence by the ions was examined.

Effect of deuterated water on the fluorescence of Tb^{3+} in Tb^{3+}–transferrin was studied by lyophilizing two identical solutions of Tb^{3+}–transferrin and then redissolving in H_2O for one of them and in D_2O for the other. The fluorescence intensity and lifetime were measured.

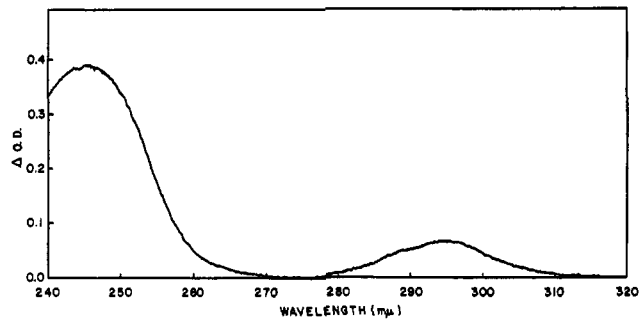

FIGURE 1: Ultraviolet difference spectrum of Tb^{3+}-saturated transferrin *vs.* metal-free transferrin. Transferrin concentration = 10^{-4} M in Tris-HCl buffer ($\mu = 0.01$, pH 8.5) and 0.005 M $NaHCO_3$. Optical path length = 1 mm.

Results

Binding of Rare Earth Ions to Transferrin. The binding of the rare earth ions to transferrin was studied by ultraviolet difference absorption spectroscopy similar to the method described by Tan and Woodworth (1969). A typical ultraviolet difference spectrum between Tb^{3+}–transferrin and transferrin taken with 1-mm ultraviolet cell is shown in Figure 1, where the concentration of the transferrin is about 10^{-4} M and the concentration of terbium ion is about 4×10^{-4} M. The spectrum has the characteristic shape of a difference spectrum due to tyrosinate residues of a protein. It has two sharp maxima in the region of 245 and 295 mμ. The shape of the difference spectra for other rare earth ions are identical; however, the optical density at 245 mμ of the difference spectrum is not the same for the different rare earth ion–transferrin complexes.

The titration curves for the rare earth ions and transferrin are shown in Figure 2, where ΔOD at 245 mμ is plotted against the number of metal ions in solution per transferrin molecule. A complete analysis of the metal binding requires data additional to these titration curves. However, drawing a tangent through the origin which makes an intercept with the extension of the horizontal part of the curve indicates that the maximum number of metal ions bound to a transferrin molecule specifically is two for Ho^{3+} and Er^{3+}; slightly less than two for Tb^{3+} and Eu^{3+}; and one for Nd^{3+} and Pr^{3+}. A Scatchard plot of the titration curves (assuming that the maximum change of optical density at 245 mμ is equivalent to the maximum number of binding sites) shows that the relationship between $1/\bar{\nu}$ and $1/c$ is not linear ($\bar{\nu}$ is the average number of ions associated with each protein and c is the concentration of rare earth ion.) This indicates that the two metal binding sites are inequivalent. Thus, it is fair to say that there are two specific binding sites for Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+}, and that there is only one specific binding site for Nd^{3+} and Pr^{3+}. ΔOD at 245 mμ for maximum binding is 0.333 ± 0.005 for Tb^{3+}–transferrin, 0.28 ± 0.01 for Eu^{3+}–transferrin, 0.333 ± 0.005 for Ho^{3+}–transferrin and also for Er^{3+}–transferrin, 0.150 ± 0.005 for Nd^{3+}–transferrin, and 0.12 ± 0.01 for Pr^{3+}–transferrin.

Fluorescence Properties of the Trivalent Lanthanide Ions and Transferrin. When Tb^{3+} was bound to transferrin, Tb^{3+} fluo-

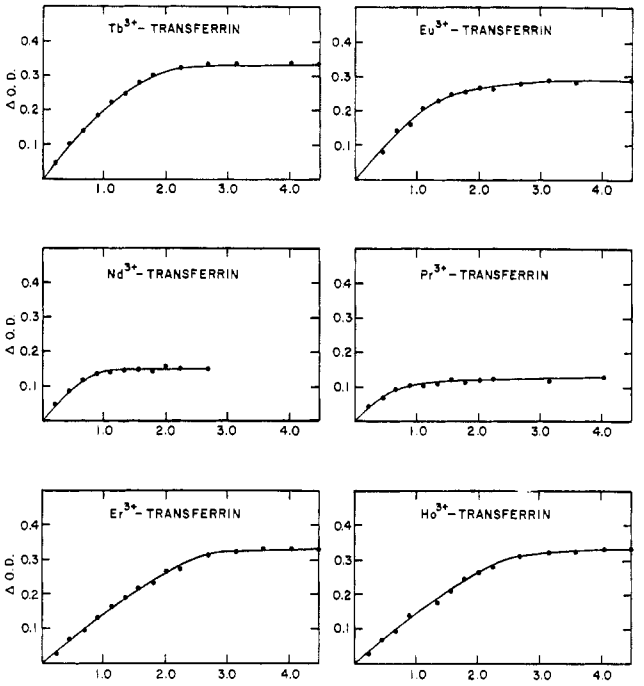

FIGURE 2: Titration of transferrin with Tb^{3+}, Eu^{3+}, Nd^{3+}, Pr^{3+}, Er^{3+}, and Ho^{3+}. The abscissa is total moles of metal ions per total moles of transferrin and the ordinate is the changes in optical density at 245 mμ. Transferrin concentration = 0.89×10^{-5} M in Tris-HCl buffer (μ = 0.01, pH 8.5) and 0.005 M $NaHCO_3$. Optical path length = 1 cm.

rescence intensity was enhanced tremendously (by a factor $\sim 10^5$) when excited at 295 mμ; however, when excited at 352 mμ the Tb^{3+} fluorescence can hardly be observed for the same sample. Its fluorescence spectrum is just very slightly altered.

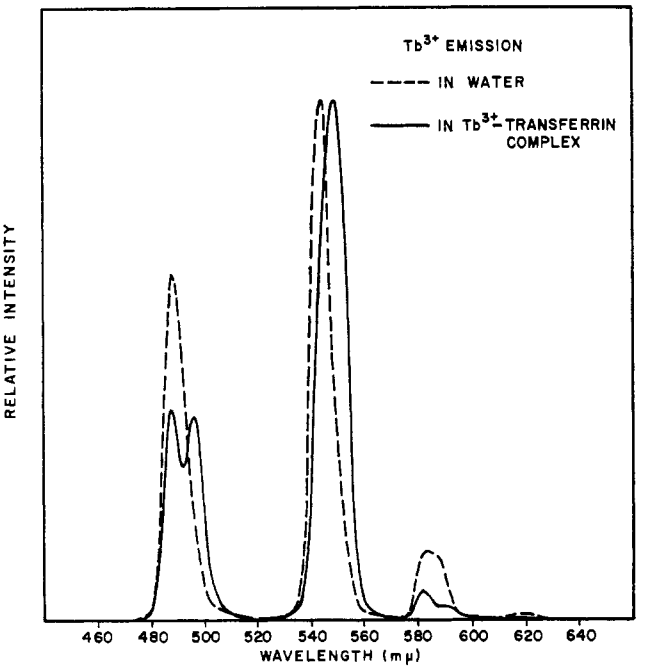

FIGURE 3: Comparison of fluorescence spectra of Tb^{3+} in water and in Tb^{3+}–transferrin complex. λ_{ex} = 295 mμ. In the figure, the Tb^{3+} emission intensity in water has been increased by a factor of about 10^5 in order to compare its spectral shape with that of the Tb^{3+}–transferrin complex on the same plot.

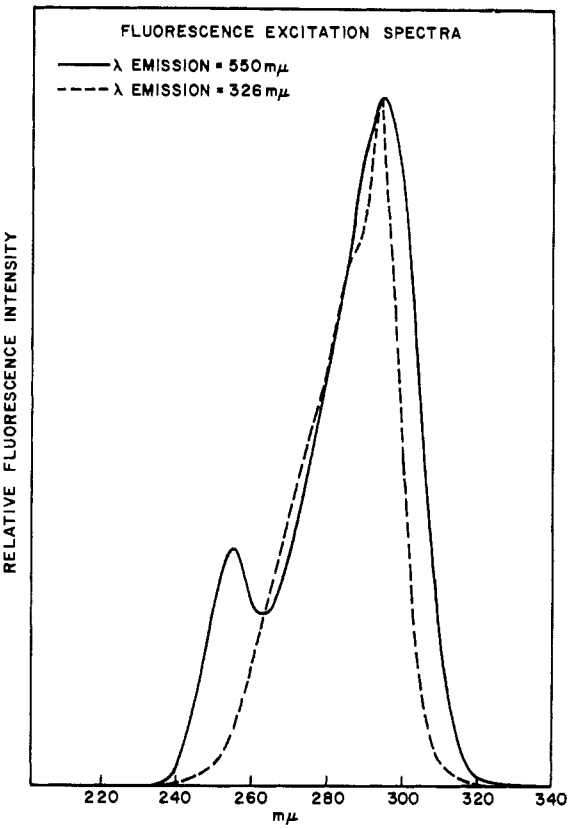

FIGURE 4: Comparison between the fluorescence excitation spectrum of Tb^{3+} bound to transferrin (λ_{em} 550 mμ) and the fluorescence excitation spectrum of transferrin (λ_{em} 326 mμ). Transferrin concentration = 0.89×10^{-5} M.

Tb^{3+} fluorescence spectra in water and in Tb^{3+}–transferrin solution are shown in Figure 3. When Eu^{3+} was bound to transferrin, Eu^{3+} fluorescence was not enhanced regardless of the wavelength of excitation. The fluorescence excitation spectrum of Tb^{3+} (concentration $\sim 10^{-5}$ M) bound to trans-

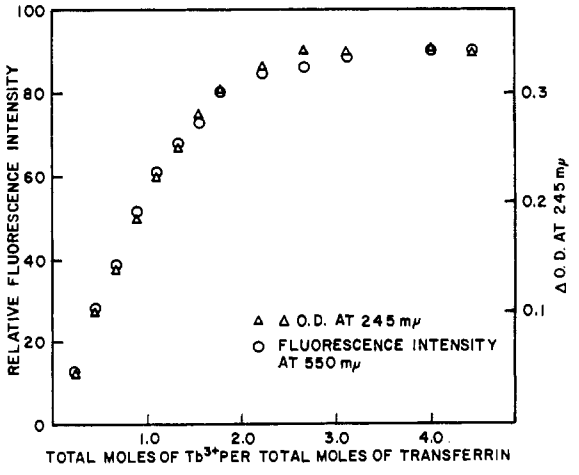

FIGURE 5: Comparison of the titration curves of transferrin with Tb^{3+} done by ultraviolet difference spectroscopy and by spectrofluorometric spectroscopy. Transferrin concentration = 0.89×10^{-5} M in Tris-HCl buffer (μ = 0.01, pH 8.5) and 0.005 M $NaHCO_3$. Optical path length = 1 cm. λ_{ex} = 295 mμ for fluorescence measurement.

TABLE I: Fluorescence Lifetimes of Tb^{3+} in Tb^{3+}–Transferrin Complexes.

Sample	Run	Rate	Std Dev	Lifetime (msec)
Tb^{3+}–transferrin	1	0.7872	0.0073	1.27
Tb^{3+}: 50% saturation	2	0.7869	0.0066	1.27
	3	0.7933	0.0046	1.26
Tb^{3+}–transferrin	1	0.7635	0.0064	1.31
Tb^{3+}: 100% saturation	2	0.7604	0.0072	1.31
Tb^{3+}–transferrin–Fe^{3+}	1	0.7990	0.0047	1.25
Tb^{3+}: 50% saturation	2	0.8023	0.0047	1.25
Fe^{3+}: 50% saturation				
Tb^{3+}–transferrin–Cu^{2+}	1	0.7775	0.0176	1.29
Tb^{3+}: 50% saturation	2	0.7820	0.0145	1.28
Cu^{2+}: 50% saturation	3	0.7832	0.0160	1.28

TABLE II: Estimation of the Number of Tyrosyl Residues Involved in Binding of the Trivalent Lanthanide Ions per Transferrin Molecule.

Ions	$\Delta\epsilon_{245m\mu} \times 10^{-3}$	No. of Tyr. Residues[a]
Pr^{3+}	13.4 ± 2	1.34 ± 0.2[b]
Nd^{3+}	17 ± 2	1.7 ± 0.2
Eu^{3+}	31.6 ± 2	3.16 ± 0.2
Tb^{3+}	37.5 ± 2	3.75 ± 0.2
Ho^{3+}	37 ± 2	3.7 ± 0.2
Er^{3+}	37 ± 2	3.7 ± 0.2

[a] $\Delta\epsilon_{245m\mu}$ per tyrosyl residue is taken to be 10^4. [b] Estimated precision.

ferrin and the fluorescence excitation spectrum of the amino acid of the transferrin were shown in Figure 4. It is clear that they are different and the shape of the Tb^{3+} fluorescence excitation spectrum is similar to the difference ultraviolet absorption spectrum due to tyrosinate residues shown in Figure 1. On the other hand, the shape of the Tb^{3+} fluorescence excitation spectrum in Tb^{3+}–transferrin (concentration $\sim 10^{-5}$ M) is quite different from the shape of Tb^{3+} fluorescence excitation spectrum in water.

The amino acid fluorescence of the transferrin was slightly quenched (~8%/site) by the binding of Tb^{3+} ions. The binding of Tb^{3+} with transferrin was also studied by fluorometric titration. The result is shown in Figure 5. It is identical with the titration curve by ultraviolet difference spectroscopy also compared in Figure 5.

The fluorescence lifetime of Tb^{3+} bound to transferrin is ~1.27 msec. This is significantly longer than the Tb^{3+} fluorescence lifetime (0.432 msec) measured in water. The deuterium solvent effect shows that the fluorescence intensity and the fluorescence lifetime of Tb^{3+} in Tb^{3+}–transferrin in D_2O were increased by 50% over that in H_2O.

Results of the experiment on the relative binding strength of the lanthanide ions are given as follows in the order of decreasing binding strength: $Er^{3+} \gtrsim Ho^{3+} \gtrsim Tb^{3+} > Eu^{3+} > Nd^{3+} \approx Pr^{3+}$.

Distance between the Two Specific Binding Sites. Results on measurement of the effect of Er^{3+}, Ho^{3+}, or Eu^{3+} bound to transferrin at one site on the Tb^{3+} (bound to the other metal binding site of the transferrin) fluorescence show that there is no measurable quenching of Tb^{3+} fluorescence intensity by the other ions within experimental uncertainty and that the fluorescence lifetime of Tb^{3+} is not reduced either. The transfer of excitation energy of Tb^{3+} bound to one metal-binding site of the transferrin to Cu^{2+} or Fe^{3+} bound to the other metal binding site was not observed either within experimental uncertainty. Results on the lifetime measurement on Tb^{3+}–transferrin and Tb^{3+}–transferrin–Fe^{3+} were shown in Table I.

Discussion

Binding Properties of the Trivalent Lanthanide Ions to Transferrin. It is well established that transferrin has two specific metal binding sites for Fe^{3+}, Cu^{2+}, Cr^{3+}, Mn^{3+}, Co^{3+}, and Ga^{3+} (Aisen *et al.*, 1969; Aasa *et al.*, 1963). Our results on the bindings of the trivalent lanthanide ions to transferrin by ultraviolet difference absorption and fluorescence spectroscopy show that there are two specific metal-binding sites per transferrin molecule for Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+} and that there is only one specific binding site per transferrin molecule for Nd^{3+} and Pr^{3+}. The chemistry of the lanthanide ions is very similar; however, they differ in their ionic radii. The ionic radius of the metal ions is: Pr^{3+}, 1.013 Å; Nd^{3+}, 0.995 Å; Eu^{3+}, 0.950 Å; Tb^{3+}, 0.923 Å; Ho^{3+}, 0.894 Å; and Er^{3+}, 0.881 Å (Cotton and Wilkinson, 1962). Thus a possible explanation for the observed results is that the size of the metal-binding sites is rather small, and the two sites are not equivalent for the binding of very large metal ions. Although it contradicts the observation for Fe^{3+}–transferrin studies, the evidence supporting our view is: (1) the ionic radius of ferric ion is much smaller than the ionic radii of the rare earth ions; (2) physiological studies (Fletcher and Huehns, 1968) of Fe^{3+} uptake from transferrin by reticulocytes suggest a difference between two iron binding sites; and (3) in Cr^{3+}–transferrin complexes the Cr^{3+} ions at the two binding sites have somewhat different spin Hamiltonians (Aisen *et al.*, 1969).

Ultraviolet difference spectra between the trivalent lanthanide ion–transferrin and transferrin show the characteristic difference absorption spectra of tyrosinate residue of a protein, *i.e.*, it has two peaks, one in 245-mμ region and the other in 295-mμ region. The result indicates possible involvement of tyrosyl residues as ligands in the metal binding. More direct evidence of the involvement in binding by tyrosyl residue by fluorescence measurement will be discussed later.

Assume that the tyrosyl residues are directly involved in the metal binding, we may approximately estimate the number of tyrosyl residues involved in the metal-binding site from the ΔOD at 245 mμ shown in Figure 2 and by taking the value of $\Delta\epsilon_{245m\mu} \approx 10{,}000$ per tyrosyl residue which is quite reasonable since the conditions under which the experiment was carried out are similar to those given by Tan and Woodworth (Tan and Woodworth, 1969). Although the estimation is approximate, the analysis here is sufficient for the discussion on Tb^{3+} in the next section. The results are shown in Table II. It is obvious then that there are two tyrosyl residues involved in each of the binding sites of Tb^{3+}, Ho^{3+}, and Er^{3+}, and one would speculate that one of the Eu^{3+}-binding sites involve two tyrosyl residues and the other Eu^{3+}-binding site has only one tyrosyl residue as ligand. For Nd^{3+} and Pr^{3+}, only one

specific metal-binding site is proposed and they are only weakly bound to transferrin. Less than two tyrosyl residues were involved in the binding of Nd^{3+} and Pr^{3+}.

Fluorescence Properties of the Trivalent Lanthanide Ions and the Transferrin. When Tb^{3+} is bound to transferrin, Tb^{3+} fluorescence increased tremendously. The fluorescence titration curve shown in Figure 5 indicates that only those Tb^{3+} bound to the specific metal-binding sites of a transferrin will enhance their emission. When Tb^{3+}–transferrin was excited at 295 mμ, the estimated order of magnitude of increase of Tb^{3+} fluorescence intensity is about 10^5. However, when it was excited at 352 mμ no enhancement of Tb^{3+} fluorescence intensity (within order of magnitude) was observed upon binding to transferrin. The lifetime of the Tb^{3+} fluorescence increased from 0.432 msec in H_2O to 1.27 msec when bound to transferrin. The nonproportional increase in the fluorescence intensity and lifetime is probably the result of transfer of excitation energy of the protein to bound Tb^{3+} and dissipated by its fluorescence. The increase of Tb^{3+} fluorescence lifetime when it is bound to transferrin is probably the result of the different environment that the Tb^{3+} is in. It is very clear that Tb^{3+} bound to transferrin is in a less symmetrical ligand field than Tb^{3+} free in water.

The possible source of the excitation energy that transferred to Tb^{3+} is the tyrosyl residues that are involved in the binding of Tb^{3+}. The fluorescence excitation spectrum of Tb^{3+} bound to transferrin with emission observed at 550 mμ shown in Figure 4, is similar to the ultraviolet difference absorption spectrum shown in Figure 1 which is due to dissociated tyrosyl residues of the transferrin. Note here that the fluorescence excitation spectrum of Tb^{3+} bound to transferrin is not the same as the absorption spectrum of Tb^{3+}. The reason for this discrepancy is that the excitation spectrum shown in Figure 4 is the result of the Tb^{3+} excitation spectrum plus energy transfer, where the Tb^{3+} excitation spectrum is negligible.

Taking the value of $\Delta\epsilon_{295m\mu}$ equal to 2350 per dissociated tyrosyl residue (Tan and Woodworth, 1969) and assume two tyrosyl residues involved in the Tb^{3+} binding, the ratio of $\Delta\epsilon_{295m\mu}$ of transferrin per Tb^{3+} binding site to $\epsilon_{295m\mu}$ of Tb^{3+} is about 5×10^4. If we multiply 5×10^4 by 2.9 (ratio of fluorescence lifetime of Tb^{3+} bound to transferrin to Tb^{3+} in H_2O), the result is very close to the enhancement of Tb^{3+} fluorescence intensity due to binding to transferrin. Therefore, if the light absorbed by the tyrosyl residues involved in the binding of Tb^{3+} is very efficiently transferred to the bound Tb^{3+}, the Tb^{3+} fluorescence will be enhanced tremendously. In order to have such efficient energy transfer, it is very likely that the said tyrosyl residues are directly bound to Tb^{3+}.

When Eu^{3+} was bound to transferrin, no enhancement of Eu^{3+} fluorescence was observed. A case in which organic molecules only sensitized Tb^{3+} emission but not Eu^{3+} emission has been observed (Heller and Wasserman, 1965). A possible explanation for this discrepancy is that the energy level of the donor is much closer to the energy level of Tb^{3+} than the energy level of Eu^{3+}.

The binding of Tb^{3+} to transferrin slightly quenches transferrin fluorescence (~8% per site). The protein fluorescence is mainly from tryptophan residues. There are nine tryptophan residues in transferrin (Aisen, 1971). Thus, it is possible that a tryptophan residue of low fluorescence quantum yield is near the Tb^{3+}-binding site and the tryptophan fluorescence is quenched by tyrosinate residues at the binding sites. (The critical distance of energy transfer for tryptophan–tyrosinate pair is about 13 Å.) Detailed analysis on whether the tryptophan is at the metal-binding site has not been carried out.

Studies on deuterium solvent effect on the Tb^{3+} fluorescence in Tb^{3+}–transferrin complex indicate that only very few water molecules are involved in the binding sites. A semiquantitative estimation of number of water molecules involved in the metal binding can be done by assuming that the quantum yield of Tb^{3+} in D_2O is one and by solving

$$Y(H_2O) = \frac{\omega_{D_2O}}{\omega_{D_2O} + 10\omega_{NR}}$$

$$Y(D_2O\text{–transferrin}) = \frac{\omega_{D_2O}}{\omega_{D_2O} + \omega(x)}$$

$$Y(H_2O\text{–transferrin}) = \frac{\omega_{D_2O}}{\omega_{D_2O} + \omega(x) + n_{H_2O}\omega_{NR}}$$

where $Y(H_2O)$, $Y(D_2O$–transferrin), and $Y(H_2O$–transferrin) are the quantum yields of Tb^{3+} in H_2O, in Tb^{3+}–transferrin complex in D_2O, and in Tb^{3+}–transferrin complex in H_2O; ω_{D_2O}, ω_{NR}, and $\omega(x)$ are the rate of dissipation of Tb^{3+} excitation energy by Tb^{3+} fluorescence, water vibration, and other mechanisms; 10 is the number of water molecules in the solvation shell of Tb^{3+} in aqueous solution (Templeton and Dauben, 1954), and n_{H_2O} is the number of water molecules involved in the metal binding sites of the transferrin. The ratio of quantum yields of Tb^{3+} in D_2O to that in H_2O is 9 and fluorescence lifetime of Tb^{3+} in H_2O is 0.432 msec n_{H_2O}, thus found, is equal to 1.4.

Distance between the Two Specific Metal-Binding Sites. The transfer of excitation energy between rare earth ions has been investigated in detail in glasses. Nakazawa and Shionoya (1967) have determined the critical distance of transfer between Tb^{3+} and Eu^{3+}, Ho^{3+}, and Er^{3+}, etc., and found that the mechanism of transfer in this particular case is dipole–quadrupole resonance transfer due to the fact that dipole transitions are forbidden for the intraconfigurational transitions of 4f electrons. The critical distance and mechanism for the transfer of excitation energy for pair of ions in transferrin are not expected to vary very much since the 4f electrons of the lanthanide ions are very well shielded from surroundings.

The results of the measurement on the fluorescence intensity and fluorescence lifetime show that Tb^{3+} fluorescence is not quenched by the presence of Eu^{3+}, Ho^{3+}, or Er^{3+}. We have shown that there are two specific metal-binding sites on transferrin and their binding strength for four of the lanthanide ions are comparable. Therefore, we expect that the distribution of the ions on the two binding sites of transferrin is random. Studies done by other research workers (Aisen *et al.*, 1967; Fletcher and Huehns, 1968) also support this assumption. Although the possibility that a transferrin molecule only binds two identical metal ions is not strictly ruled out, it is very unlikely. Thus, we expect that the distance between the two binding sites must be much greater than the critical transfer distance which is in the order of 10 Å. Within the estimated experimental uncertainty of 2%, the separation between the two metal-binding sites must be greater than 16 Å. This result is quite in line with the result from electron spin resonance studies (Aasa *et al.*, 1963) showing that the two sites must be greater than 9 Å apart.

Further studies on the separation of the two metal-binding sites were carried out with Tb^{3+} as donor and Fe^{3+} or Cu^{2+} as acceptor since Fe^{3+} and Cu^{2+} have a huge molar extinction coefficient where Tb^{3+} fluoresces. The transfer of excitation energy between Tb^{3+} and Fe^{3+} is assumed to be a result of

dipole–dipole interaction and the critical distance of transfer is evaluated by well-known equations. The overlap integral between Tb^{3+} fluorescence and Fe^{3+} absorption was evaluated from Figure 6, where the fluorescence spectrum of Tb^{3+} is not corrected for spectral response of the instrument used. $\overline{K}^2$ is taken to be two-thirds and the fluorescence quantum yield is estimated to be 0.24 ± 0.06. The distance of separation for 50% transfer efficiency, R_0 thus calculated, is about 27 ± 1 Å.

The results of fluorescence lifetime measurements (Table I) show that the fluorescence lifetime of Tb^{3+} in Tb^{3+}–transferrin –Fe^{3+} complex is ~5% less than the fluorescence lifetime of Tb^{3+} in Tb^{3+}–transferrin complex. If this discrepancy is solely due to energy transfer, the separation, R, between the two specific metal-binding sites is calculated from the equation: $T = R_0^6/(R^6 + R_0^6)$, to be 43 Å apart. However, the difference between the fluorescence lifetimes of Tb^{3+} in Tb^{3+}–transferrin–Fe^{3+} complex and in Tb^{3+}–transferrin may not solely be due to energy transfer, it can be the result of slight conformational change about the binding sites as evidenced by the difference in the fluorescence lifetime of Tb^{3+} in 50% Tb^{3+}-saturated and 100% Tb^{3+}-saturated transferrin complexes. Thus, the separation between the two binding sites is equal to or greater than 43 Å.

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Addendum

Similar results were also obtained for conalbumin.

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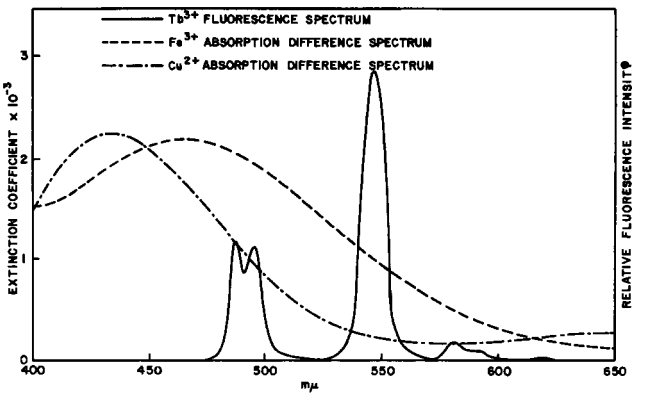

FIGURE 6: Difference absorption spectra for Fe^{3+}– and Cu^{2+}–transferrin and fluorescence spectrum of Tb^{3+} bound to transferrin. Exciting wavelength was 295 mμ for Tb^{3+} fluorescence.

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