Distance between Metal-Binding Sites in Transferrin: Energy Transfer from Bound Terbium(III) to Iron(III) or Manganese(III)†

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ABSTRACT: The distance between the two metal-binding sites of human serum transferrin has been studied by observing energy transfer between an excited terbium ion bound at one site and a ferric (or manganic) ion bound at the other site of the same transferrin molecule. From the observed reduction in terbium lifetime (relative to that of terbium transferrin), it is concluded that the intersite distance is 3.55 ± 0.45 nm. This distance is reconciled with two conflicting earlier reports that the separation between sites is greater than 4.3 nm [Luk, C. K. (1971) *Biochemistry 10*, 2838–2844] or is equal to 2.5 ± 0.2 nm [Meares, C. F., & Ledbetter, J. E. (1977) *Biochemistry 16*, 5178–5180]. The difficulty of accurately measuring the quantum yield of protein-bound terbium provides the principal source of uncertainty in these measurements.

Human serum transferrin, a monomeric glycoprotein of M_r 81 000 (MacGillivray et al., 1977), is an iron transport protein [see reviews by Aisen & Brown (1977) and Chasteen (1977)]. Many other metal ions including Mn(III), Co(III), Cr(III), Cu(II), Tb(III), and Eu(III) (Tomimatsu et al., 1976; Aisen et al., 1969; Luk, 1971) can bind to the transferrin metal-binding sites. Though the protein is a single polypeptide chain, its amino acid sequence shows that it is composed of two homologous domains, each with an iron-binding site that may have evolved through gene duplication and fusion. Why are there two sites? Do they have different biological roles? Do they interact? In answering these questions, it may be useful to know the distance between the two sites. Since they are more than 1 nm apart (Aasa et al., 1963; Windle et al., 1963), this may be determined by measuring the efficiency of energy transfer between an excited $^5D_4$ Tb(III) ion bound at one site and a Fe(III) or Mn(III) ion bound at the other. However, we have found that the earlier studies by Luk (1971) and Meares & Ledbetter (1977) suffered from systematic experimental errors. We have jointly resolved these difficulties by measuring terbium luminescence lifetimes in protein samples which were nearly saturated with iron(III) or manganese(III).

Experimental Procedures

Iron-free human serum transferrin (electrophoretic purity 98–99%) was purchased from Calbiochem-Behring and used without further purification; sodium dodecyl sulfate ($NaDodSO_4$)[1] gel electrophoresis (Makey & Seal, 1975) of apotransferrin revealed a single band. Protein concentrations were determined spectrophotometrically by assuming $A_{280}^{1\%} = 10.9$ for the apoprotein (Harris & Aisen, 1975). All reagents were the purest commercially available. Standard terbium chloride solutions were prepared from $TbCl_3 \cdot 6H_2O$ (Alfa Inorganics, 99.9% pure), which was vacuum desiccated before weighing. Buffers used were 10 mM in either Tris or Hepes and 25 mM in sodium bicarbonate. Standard 4 mM ferric NTA (two NTA molecules per iron, pH 3.5) and ferric citrate solutions (four citrates per iron, pH 3.5) were prepared from iron wire and calibrated with ferrozine (Harris & Aisen, 1975). When apotransferrin samples were titrated with ferric NTA (monitored by the absorbance at 465 nm), the results always indicated a metal-binding capacity of at least 1.8 ferric ions per transferrin. Apoprotein samples were also readily titrated with ferric citrate with similar results. To avoid contamination by extraneous metal ions, we had previously washed all labware with mixed HNO_3/H_2SO_4 (Thiers, 1957), doubly deionized and distilled H_2O was used for buffers, and only fresh solutions were studied.

A Perkin-Elmer/Hitachi MPF2A or a Perkin-Elmer MPF 3L fluorescence spectrophotometer was used to record fluorescence spectra (uncorrected for instrumental response). A Gilford 250 or a Cary 14 UV visible spectrophotometer was used to measure protein concentrations at 280 nm and monitor iron titrations at 465 nm.

Lifetime Measurements. The equipment used by C.F.M. and S.M.Y. in measuring fluorescence lifetimes, described elsewhere (Thomas et al., 1978), was generously made available by Professor Lubert Stryer. A Chromatix CMX-4 dye laser with Rhodamine 6G, frequency doubled to provide a pulsed excitation source at 300 nm, was used by P.O. and R.B. In different experiments, transferrin samples were irradiated by pulsed laser light at 257, 300, and 488 nm. The decay of excited terbium with time was monitored by observing its emission at 550 nm and recorded by a Hewlett-Packard multichannel scaler. The detection chamber was maintained at 25 °C since it was found that near room temperature the intensity increased 2.5%/°C. Linear least-squares analysis of data (single exponential plus flat baseline) was used in calculation of the slope in semilogarithmic plots of fluorescence intensity vs. time (Thomas et al., 1978). The measured lifetimes were found to be independent of the excitation wavelength.

Iron–Terbium Transferrin. Iron–terbium transferrin samples were prepared by adding solutions of $Fe(NTA)_2$ to the apoprotein in 10 mM Hepes and 25 mM HCO_3^-, pH 8.0. With $Fe(NTA)_2$ (Bates & Schlabach, 1973), stoichiometric amounts of iron could be added to the protein to produce 50, 70, 75, 80, 85, and 90% saturation levels corresponding to

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonate; NTA, nitrilotriacetate; $NaDodSO_4$, sodium dodecyl sulfate.

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metal/protein ratios of 1/1 through 1.8/1. After incubation for 15 min, NTA was removed either by 48-h dialysis (four changes of outer buffer, each having a volume 250 times the volume of the sample) or by column purification, first through Sephadex G-25 resin equilibrated with $NaClO_4$ and second through G-25 resin equilibrated with buffer. Freshly prepared terbium chloride stock solution was added to produce 10% saturation, and after being gently mixed for approximately 2 min, the sample was ready for lifetime measurements. For excitation at 257, 300, and 488 nm, protein concentrations were 10^{-5}, 10^{-7}, and 10^{-4} M, respectively. It is necessary to remove NTA because our studies have shown NTA competitively binds terbium and also changes the observed lifetime of transferrin-bound terbium when left in solution [presumably due to displacement of coordinated water molecule(s) around terbium ion in a Tb–transferrin–NTA ternary complex]. Both dialysis and column chromatography appeared to remove NTA; however, we found minimum dilution and less contamination by extraneous metal ions when dialysis was used. Terbium transferrin lifetimes were within 2% of each other regardless of whether or not the apotransferrin used for simple preparation had been dialyzed previously for 48 h.

Manganese–Terbium Transferrin. Manganese sulfate or chloride stock solutions were freshly prepared from the vacuum-desiccated light pink monohydrate powders. Apotransferrin solution was prepared in 10 mM Tris and 25 mM HCO_3^- buffer, pH 8.9. Aliquots of manganese stock solution were added to protein solutions in 1/1 through 1.8/1 mole ratios. These samples, and an apotransferrin blank, were oxidized by either peroxide or oxygen (Inman, 1956). For peroxide oxidation, each sample was treated with 0.15% hydrogen peroxide at 4 °C for 2 days. For oxidation by oxygen, samples were degassed via aspirator vacuum and exposed to pure O_2 for several minutes while stirring. This process was repeated 3 times, and the sample was then allowed to stand for 48 h at 4 °C. Some samples were dialyzed for 2 days against 10 mM Hepes and 25 mM HCO_3^-, pH 8.0 (four changes of 250 volumes); samples used without this purification gave similar results. Terbium was then added to produce 10% saturation, and the lifetimes were measured within 30 min. Mn(III) transferrins were assayed for percent Mn(III) binding by visible absorbance measurements using a molar absorptivity of 8.7×10^3 M^{-1} cm^{-1} at 429 nm (Inman, 1956).

Diferric Transferrin and Diterbium Transferrin. Diterbium transferrin (10^{-4} M) was freshly prepared by saturation of apotransferrin (in 25 mM $NaHCO_3$ and 10 mM Hepes, pH 8.0) with terbium chloride stock solution. The terbium lifetime was measured, using 488-nm excitation. As a check for intermolecular energy transfer, the lifetime of a diterbium transferrin sample which contained ~0.4 mM differric transferrin was also measured and found to be identical within experimental error (2%) to the unperturbed lifetime.

Reduction of Fluorescence of Intrinsic Transferrin Tryptophans by Titration with Ferric NTA or Ferric Citrate. Apotransferrin solutions (17 μM) were prepared in 25 mM $NaHCO_3$ buffer, pH 8.3. The samples were excited at 288 nm while the protein emission spectrum was recorded from 300 to 400 nm. After addition of each aliquot of 3.5 mM $Fe(NTA)_2$ (or of 3.5 mM ferric citrate), the sample was slowly and carefully mixed before the emission spectrum was recorded. Results are shown in Figure 4.

Lifetime of Terbium Transferrin in D_2O. A terbium–transferrin solution (1 μM transferrin, 10% saturated with terbium) in D_2O buffer (25 mM $NaHCO_3$, 10 mM Hepes, and 100% D_2O, pD 8.0) was prepared by 1/100 dilution of 100 μM Tb–transferrin/H_2O buffer (25 mM $NaHCO_3$ and 10 mM Hepes, pH 8.0). The diluted solution was excited at 300 nm, and decay rates were measured at 550 nm. To insure against any significant H_2O contamination, it was necessary that the sample be lyophilized twice from D_2O buffer and redissolved in fresh D_2O buffer, and the measurement was repeated. Both experiments yielded a lifetime of 1.79 ms, which may be compared to the lifetime of 1.21 ms observed in the H_2O solution of the same sample. This agrees with the previously reported (Luk, 1971) finding that the terbium transferrin lifetime in D_2O increased by a factor of 1.5 over that in H_2O. According to the results of Horrocks & Sudnick (1979), this is consistent with the possibility that one H_2O molecule is coordinated to transferrin-bound terbium.

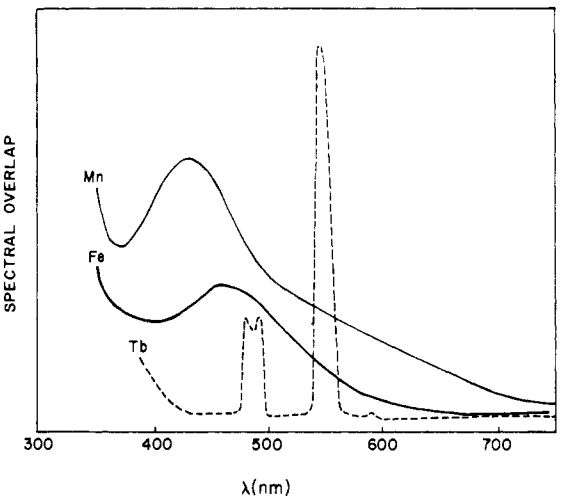

FIGURE 1: Spectral overlap between terbium–transferrin emission (dotted line) and iron–transferrin and manganese–transferrin absorptions (solid lines).

Results

The general features of energy transfer from a donor to a distant acceptor were originally worked out by Förster (1948). Transfer of energy depends on the overlap of the emission spectrum of a donor, measured by its fluorescence $F_0(\lambda)$, with the absorption spectrum of an acceptor, measured by the extinction coefficient $\epsilon(\lambda)$. The spectral overlap integral J is determined by

$$J = \frac{\int F_0(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda}{\int F_0(\lambda) \, d\lambda} \quad (1)$$

Figure 1 illustrates the spectral overlap between terbium emission and iron–transferrin and manganese–transferrin absorption.

R_0, the distance at which 50% energy transfer is achieved, can be calculated from

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6} \times 9.7 \times 10^2 \text{ nm} \quad (2)$$

The orientation factor κ^2 measures the angular orientation of one dipole with respect to the other. Here, κ^2 is given its average value of $^2/_3$ due to the effective isotropic symmetry of the electronic transitions of both donor and acceptor. The quantum yield, Q_0, is estimated to be between 0.25 and 0.68 as discussed below; experimental limitations preclude the direct measurement of Q_0. The index of refraction, n, of the intervening medium is taken to lie between 1.33 and 1.50. By use of the limiting values for Q_0 and n, R_0 for terbium → iron transfer was calculated to be between 2.47 and 3.07 nm; R_0

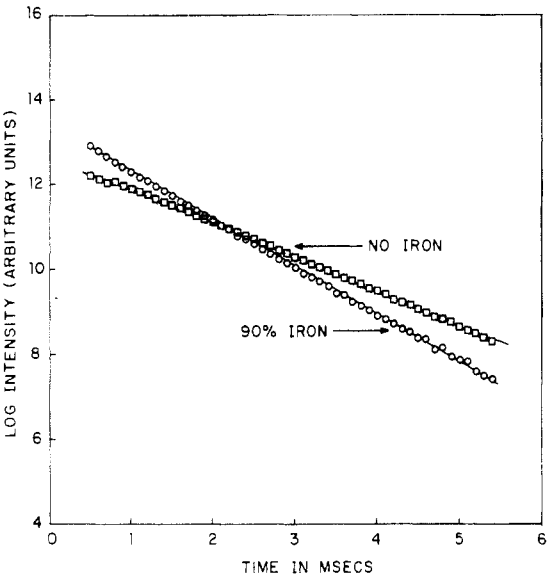

FIGURE 2: Natural log of fluorescence decay curves generated from 550-nm emission data. Terbium–transferrin (10%) without iron (□) and with 90% iron (○). Buffer was 10 mM Hepes and 25 mM $NaHCO_3$, pH 8.0. Laser excitation wavelength was 300 nm. Samples without iron were ≃0.1 μM in protein; samples with iron were ≃10 μM in protein.

Table I: Decay of Various Fe–Tb–Transferrins[a]

x–10% Tb–Tf, x =	decay rate, k (s^{-1})	lifetime (ms)	% error
no Fe	831[b]	1.20	0.14
no Fe	812[c]	1.23	
50% Fe	900[b]	1.11	0.18
65% Fe	900[c]	1.11	
70% Fe	964[c]	1.04	
75% Fe	1009[b]	0.99	0.22
80% Fe	1021[c]	0.98	
85% Fe	1017[c]	0.98	
90% Fe	1025[b]	0.98	0.12
90% Fe	1042[c]	0.96	

[a] Buffer used was 25 mM HCO_3^- and 10 mM Hepes, pH 8.0. [b] Data from R.B. and P.O. [c] Data from C.F.M. and S.M.Y.

for the terbium → manganese transfer was calculated to be between 2.81 and 3.44 nm.

The efficiency of the energy transfer, E (Stryer, 1978), can be measured by

$$E = 1 - \frac{\tau}{\tau_0} = \left[1 + \left(\frac{R}{R_0}\right)^6\right]^{-1} \quad (3)$$

where τ_0 is the lifetime of the donor in the absence of acceptor and τ is the donor lifetime in the presence of acceptor. Figures 2 and 3 show typical linear graphs of ln I vs. time, whose slopes ($=-1/\tau$) are the rates of decay for terbium transferrin as compared to terbium–iron–transferrin and terbium–manganese–transferrin, respectively.

Tables I and II report the decay rates and lifetimes of the terbium in transferrin as a function of the protein's saturation with iron or manganese. Clearly a transferrin molecule with terbium bound at one site is more likely to have iron at the other site when the iron/transferrin ratio is 1.8/1 rather than 1/1. A similar statement holds for manganese. For the solutions which were at least 80% saturated with acceptor, the average efficiency of energy transfer from transferrin-bound terbium to iron was 20%; for manganese, the average efficiency was 29%. Data are given in Tables I and II. Now R, the intersite distance, can be calculated from eq 3. For Fe–Tb–transferrin, $R = 1.25R_0^{Fe}$; for Mn–Tb–transferrin, $R = 1.16R_0^{Mn}$. Thus, the intersite distance for Fe–Tb–transferrin is between 3.1 and 3.9 nm and for Mn–Tb–transferrin between 3.3 and 4.0 nm, based on the calculated ranges of values of R_0.

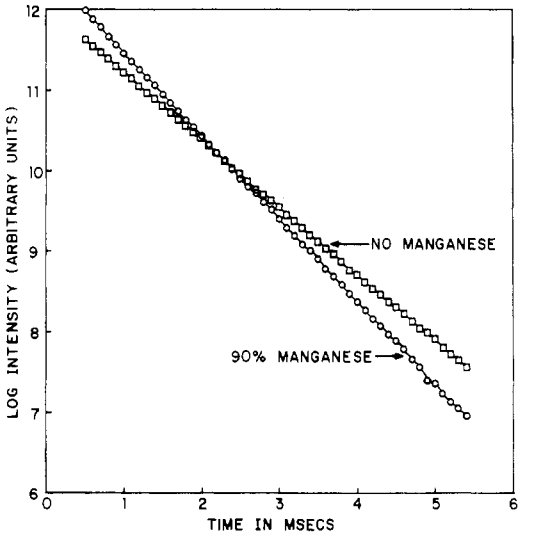

FIGURE 3: Natural log of fluorescence decay curves generated from 550-nm emission data. Terbium–transferrin (10%) without manganese (□) and with 90% manganese (○). Buffer was 10 mM Tris and 25 mM $NaHCO_3$, pH 8.9. Laser excitation wavelength was 300 nm. Samples with manganese were 0.1 μM in protein; samples with manganese were 10 μM in protein.

Table II: Decay of Various Mn–Tb–Transferrins[a]

x–10% Tb–Tf, x =	prep, H_2O_2/O_2		decay rate, k (s^{-1})	lifetime (ms)	% error
no Mn	*		802[b]	1.24	0.4
no Mn		*	801[b]	1.25	0.3
no Mn		*	811[c]	1.23	
31%		*	866[b]	1.16	0.1
36% Mn	*		883[b]	1.13	0.2
48%		*	971[c]	1.03	
59% Mn		*	957[c]	1.04	
62% Mn		*	903[b]	1.11	0.1
70% Mn	*		1026[b]	0.98	0.2
83% Mn		*	1164[c]	0.86	
95% Mn	*		1115[b]	0.90	0.2
100% Mn		*	1144[c]	0.87	

[a] Buffer used was 25 mM HCO_3^- and 10 mM Tris, pH 8.9; similar results were obtained with 25 mM HCO_3 and 10 mM Hepes, pH 8.0 (see text). [b] Data from R.B. and P.O. [c] Data from C.F.M. and S.M.Y.

There is no intermolecular energy transfer observed between diterbium transferrin and diferric transferrin, since the measured lifetimes of a sample containing both species and a sample containing only diterbium transferrin differed by less than 2%.

When apotransferrin is titrated with ferric ion, a nonlinear decrease in tryptophan fluorescence from the protein is observed with increasing iron saturation. Under conditions where iron binds sequentially rather than randomly to the two sites on transferrin, the first iron bound causes a greater decrease in tryptophan fluorescence than the second. As shown in Figure 4, this is equally true whether the titrant is $Fe(NTA)_2$, which gives iron first to the "A" site on transferrin, or ferric citrate, which favors the "B" site. These results confirm that there are aromatic residues on the protein which can efficiently donate energy to iron bound at *either* site. They also indicate that such aromatic residues are not clustered near one of the metal sites.

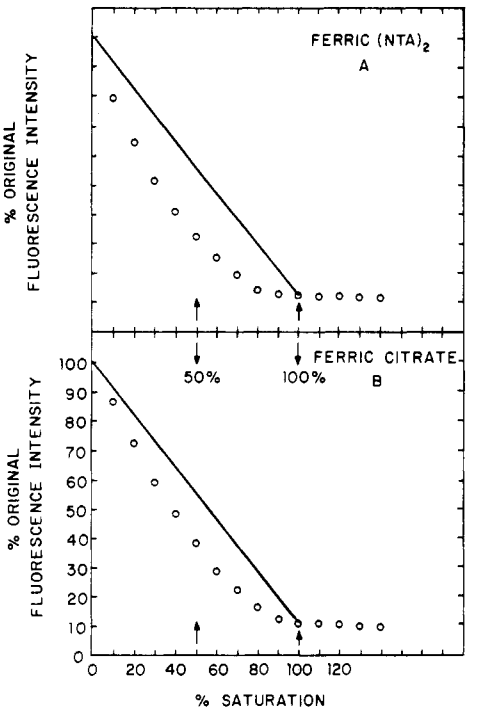

FIGURE 4: Titration curves for tryptophan emission as a function of Fe(NTA)$_2$ and ferric citrate added. Straight lines have been drawn for comparison of the deviation of the data from linearity. Note that for a given amount of added iron, the fluorescence at site A is quenched to a greater degree than that at site B.

Discussion

Error Limits. The results from both the ferric and manganic acceptors indicate that the intersite distance in transferrin is between 3.1 and 4.0 nm. Uncertainties stem from two possible sources, assumptions used to calculate R_0 and experimental errors associated with lifetime measurements. The associated error in the latter is less than 2% (Table I). According to eq 2, R_0 is directly proportional to $Q_0^{1/6}n^{-2/3}$; neither of these terms can be directly measured. The index of refraction of the medium between donor and acceptor molecules, n, can be estimated to be between 1.33 and 1.50 (Steinberg, 1971). A 0.1 error in n leads to a 4.6% error in R_0. Q_0, the quantum yield for radiative decay in the absence of acceptor species, cannot be measured due to the extremely small absorption coefficient of the lanthanide and the low solubility of the terbium–transferrin complex. Rigorous upper and lower bounds can be determined as follows:

(1) The upper bound can be obtained from the ratio of the lifetimes of Tb–transferrin in H_2O and in D_2O. This ratio is $Q_0^+ = (1.21 \text{ ms})/(1.79 \text{ ms}) = 0.68$.

(2) The lower bound on Q_0 can be obtained by assuming that the radiative lifetime, $\tau_f = k_f^{-1}$, of Tb(III) in transferrin should be no longer than the radiative lifetime of aqueous Tb(III) [4.75 ms between 10 and 65 °C (Stein & Wurzberg, 1975)]. It is reasonable to assume that the asymmetric arrangement of ligands in a transferrin metal-binding site will cause radiative transitions of bound Tb(III) to be less forbidden (shorter τ_f) than in the case of symmetrically solvated aqueous Tb(III). This provides the estimated lower bound $Q_0^- = (1.21 \text{ ms})/(4.75 \text{ ms}) = 0.25$. This is very close to the value $Q_0 = 0.25$ previously proposed by Luk (1971).

This range of values for Q_0 seems reasonable when compared with other data in the literature. For example, the radiative lifetime of Tb(III)–tris(acetylacetonate) monohydrate in ethanol has been found to be $\tau_f = 2.5$ ms (Dawson et al., 1966). Assuming, as did Horrocks et al. (1975) for Tb–thermolysin, that the k_f for Tb–transferrin has the same value, we would obtain the result $Q_0 = 0.51$. Also, six different terbium chelates (among them some amino acids) have values of Q_0 ranging from 0.157 and 0.401 (Berner et al., 1975) with an average value of 0.25.

Thus, the quantum yield of Tb–transferrin should lie between 0.68 and 0.25. An error of 0.2 in Q_0 leads to an error of 8.3% in R_0. The total error in the calculation of R_0 can be as high as 9.5% resulting in an overall uncertainty of 9.7% in R. The intersite distance is 4.0 nm using the upper bounds on Q_0 and n; the lower bounds yield 3.1 nm.

Mechanism of Energy Transfer between Metal Ions. The efficiency of energy transfer for any multipolar mechanism is given by a generalization of eq 3:

$$E = \left[1 + \left(\frac{R}{R_0}\right)^m\right]^{-1} \quad (4)$$

where $m = 6$ if the dipole–dipole interaction causes the energy transfer. Assuming that Tb ($^5D_4$) transfers its energy to Mn(III) and Fe(III) by the same mechanism, one can derive the relation

$$m = \ln\left[\left(\frac{E}{1-E}\right)_1\left(\frac{1-E}{E}\right)_2\right] \Big/ \left[\ln\frac{R_{0(1)}}{R_{0(2)}}\right] \quad (5)$$

where the subscripts 1 and 2 stand for Fe– and Mn–transferrin, respectively. m turns out to be 5.4, which is sufficiently close to 6 to be consistent with the dipole–dipole mechanism for energy transfer.

Mechanisms of Terbium Excitation. The lowest radiative level, $^5D_4$ of a bound Tb(III) ion, can be populated directly or indirectly. Direct excitation from the $^7F_6$ ground state is conveniently accomplished by the 488-nm light of an Ar^+ laser. Indirect excitation of transferrin-bound terbium may occur by at least two likely routes: UV excitation of coordinated tyrosinates (Luk, 1971) or UV excitation of nearby tryptophan(s) followed by radiationless energy transfer to terbium-coordinated tyrosinates. The relative importance of these two routes is a function of the UV wavelength used because the wavelengths of maximum absorption of tryptophan and tryosinate are respectively 280 and 295 nm. The route via tryptophan can make a substantial contribution to terbium excitation when the wavelength is 290 nm because in this range tryptophan has a larger extinction coefficient than those of tyrosine or tyrosinate [see Wetlaufer (1962)] and because values of R_0 for radiationless energy transfer from tryptophan to tyrosinate are appreciable, ranging from 0.84 (Eisinger et al., 1969) to 1.33 nm (Edelhoch et al., 1967). Other values of R_0 for energy transfer between various aromatic amino acid pairs have been tabulated in the review article by Steinberg (1971).

Metal Ion Quenching of Intrinsic Transferrin Fluorescence. While transfer of energy from an excited tryptophan to a terbium-coordinated tyrosinate residue with a critical distance R_0 of 0.84–1.33 nm is possible, energy transfer from tryptophan to an iron-coordinated tyrosinate is even more favorable, having an R_0 of 2.1–2.7 nm (Lehrer, 1969). Lehrer (1969) and Evans & Holbrook (1975) have noted that intrinsic transferrin fluorescence is sharply reduced as a function of saturation with ferric ion [Fe(NTA)$_2$] or cupric ion ($CuSO_4$ solution). Ainscough et al. (1980) have found that conalbumin behaves similarly.

Under conditions where iron binds sequentially to the two sites on transferrin, the first iron bound causes a greater decrease in tryptophan fluorescence than the second. As shown in Figure 4, this is true whether the titrant is Fe(NTA)$_2$, which donates iron to the A site on transferrin, or ferric citrate, which

favors the B site. These results confirm that there are aromatic residues on the protein that can efficiently donate energy to iron bound at *either* site. In a situation with iron bound at one site and terbium at the other, a centrally located tryptophan would be roughly $2.5^6 = 244$ times more likely to donate its energy to the iron site than to the terbium site, according to the R_0^6 values for each process.

This phenomenon has consequences not only for intensity measurements but also for lifetime measurements; terbium bound to iron-free transferrin is much more likely to be excited by UV radiation than is terbium bound to monoferric transferrin, and it is also more likely to luminesce, providing unwanted interference. We eliminated the problem of iron-free transferrin by using samples which were almost saturated with iron, as shown in Table I (and similarly for manganese, Table II).

Errors in the Previous Experiments. In principle, one could obtain the efficiency of energy transfer, E, equally well from a static measurement of fluorescence quenching ($E = 1 - I/I_0$) or from a measurement of fluorescence decay ($E = 1 - \tau/\tau_0$). If the presence of the quencher diminishes the efficiency of *excitation* of the fluorescing species, the fluorescence intensity will be diminished for two reasons: (1) the reduction of excitation efficiency and (2) the quenching process itself. Meares & Ledbetter (1977) used 288 nm to excite Tb–transferrin fluorescence. At that wavelength, excited tryptophans evidently make a contribution to the terbium excitation process. The presence of iron will then substantially quench this excitation process as discussed above. Meares & Ledbetter (1977) measured the metal–metal distance by fluorescence quenching and obtained the value 2.5 ± 0.2 nm. As they overestimated the amount of quenching which occurs, their distance was too small. Luk (1971) obtained the distance by lifetime measurement and found $R \geq 4.3$ nm. In that experiment, however, monoferric transferrin was made by addition of ferric chloride to apotransferrin, a method subsequently shown to give low yields and complicated side reactions (Bates & Schlabach, 1973). Thus, while only a maximal 5% reduction of lifetime was seen in transferrin samples supposedly 50% iron saturated, a greater reduction would have been seen if the binding was quantitative. Therefore, his value of $R \geq 4.3$ nm was too high.

Conclusions

In recent years, luminescence from lanthanide ions such as terbium and europium has been shown to possess a number of features useful for the study of chromophores or metal-binding sites in biological systems (Stryer, 1978; Thomas et al., 1978; Horrocks et al., 1975; Horrocks & Sudnick, 1979; Yeh & Meares, 1980; Meares & Rice, 1981). As the applications become widespread, it is important to examine the basic features of each experiment to look for hidden sources of error. As described in this report, we have found that lifetime measurements are less error prone than intensity measurements; but even so, lifetime measurements involving small changes are subject to interfering emission from acceptorless donors. Furthermore, since the molar absorptivity of protein-bound Tb(III) is very small ($\epsilon_{488} \approx 0.05$ M^{-1} cm^{-1}), direct measurement of its quantum yield is impractical; plausible alternatives, such as comparison with model compounds, must await corroboration by other techniques before their reliability can be assessed.

Acknowledgments

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References

Aasa, R., Malmstrom, B. G., Saltman, P., & Vannguard, T. (1963) *Biochim. Biophys. Acta 75*, 203–222.
Ainscough, E. W., Brodie, A. M., Plowman, J. E., Bloor, S. F., Loehr, J. S., & Loehr, T. M. (1980) *Biochemistry 19*, 4072–4079.
Aisen, P., & Brown, E. B. (1977) *Semin. Hematol. 14*, 31–53.
Aisen, P., Aasa, R., & Redfield, A. G. (1969) *J. Biol. Chem. 244*, 4628–4633.
Bates, G. W., & Schlabach, M. R. (1973) *J. Biol. Chem. 248*, 3228–3232.
Berner, V. G., Barnall, D. W., & Birnbaum, E. R. (1975) *Biochem. Biophys. Res. Commun. 66*, 763–768.
Chasteen, N. D. (1977) *Coord. Chem. Rev. 22*, 1–36.
Dawson, W. R., Kropp, J. L., & Windsor, M. W. (1966) *J. Chem. Phys. 45*, 2410–2418.
Edelhoch, H., Brand, L., & Wilchek, M. (1967) *Biochemistry 6*, 547–559.
Eisinger, J., Feuer, B., & Lamola, A. A. (1969) *Biochemistry 8*, 3908–3915.
Evans, R. W., & Holbrook, J. J. (1975) *Biochem. J. 145*, 201–207.
Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–57.
Harris, D. C., & Aisen, P. (1975) *Biochemistry 14*, 262–267.
Horrocks, W. deW., & Sudnick, D. R. (1979) *J. Am. Chem. Soc. 101*, 334–340.
Horrocks, W. deW., Holmquist, B. W., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4764–4768.
Inman, J. K. (1956) Ph.D. Thesis, Harvard University.
Lehrer, S. S. (1969) *J. Biol. Chem. 244*, 3613–3617.
Luk, C. K. (1971) *Biochemistry 10*, 2838–2844.
MacGillivray, T. T. A., Mendez, E., & Brew, K. (1977) in *Proteins of Iron Metabolism* (Brown, E. G., Aisen, P., Fielding, J., & Crichton, R. R., Eds.) p 133, Grune & Stratton, New York.
Makey, D. G., & Seal, U. S. (1975) *Biochim. Biophys. Acta 453*, 250–256.
Meares, C. F., & Ledbetter, J. E. (1977) *Biochemistry 16*, 5178–5180.
Meares, C. F., & Rice, L. S. (1981) *Biochemistry 20*, 610–617.
Stein, G., & Wurzberg, E. (1975) *J. Chem. Phys. 62*, 208–211.
Steinberg, I. Z. (1971) *Annu. Rev. Biochem. 40*, 83–114.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273.
Thomas, D. D., Carlsen, W. F., & Stryer, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5746–5750.
Tomimatsu, Y., Kinti, S., & Scherer, J. R. (1976) *Biochemistry 15*, 4918–4924.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.
Widle, J. J., Wiersema, A. K., Clark, J. F., & Feeney, R. E. (1963) *Biochemistry 2*, 1341–1345.
Yeh, S. M., & Meares, C. F. (1980) *Biochemistry 19*, 5057–5062.