Characterization of Transferrin Metal-Binding Sites by Diffusion-Enhanced Energy Transfer†

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ABSTRACT: The distance from the protein surface to ferric or manganic ions in the two specific metal-binding sites of human serum transferrin has been estimated by measuring energy transfer from freely diffusing terbium chelates in aqueous solution to transferrin-bound metal ions. In addition, both monoferric forms of the protein were studied, as well as the diferric complex formed by using oxalate instead of (bi)carbonate as the auxiliary anion in binding of iron(III) to transferrin. Second-order rate constants for energy transfer between electrically neutral terbium(III)–*N*-(2-hydroxyethyl)ethylenediaminetriacetate and the Fe_A, Fe_B, and Fe_2 forms of transferrin were 0.9×10^5 M^{-1} s^{-1}, 1.4×10^5 M^{-1} s^{-1}, and 2.6×10^5 M^{-1} s^{-1}, respectively (based on iron concentration). For the Fe_2 species, substitution of oxalate for (bi)carbonate has the effect of decreasing the accessibility of both electrically neutral and negatively charged terbium chelates to the protein-bound iron chromophores. Theoretical considerations of the effect of acceptor location in the protein on energy transfer suggest that the iron chromophores are not on the surface of the protein but are <1.7 nm below the surface. The use of diterbium transferrin as energy donor to a small cobalt chelate in solution or to diferric transferrin corroborates these results.

Human serum transferrin is a monomeric protein of M_r 81 400 (MacGillivray et al., 1977). Transferrin has two specific sites for binding of metal ions; binding of a metal ion such as Fe(III) must be accompanied by binding of an anion such as carbonate or bicarbonate (Aisen & Brown, 1977; Chasteen, 1977). From sedimentation studies, the shape of the protein is roughly that of a prolate ellipsoid with dimensions of 4-nm width and 12-nm length (Bezkorovainy & Rafelson, 1964).

The chief physiological function of human serum transferrin is iron transport, involving reversible binding of ferric ions. Therefore, it is important to characterize the location and properties of the two metal-binding sites. A new way of looking at this problem was suggested to us by the experiments of Thomas et al. (1978) involving energy transfer from long-lived excited donors to freely diffusing energy acceptors in solution. We have measured second-order rate constants for energy transfer from small terbium chelates to bound metal ions in transferrin. These rate constants provide information about the accessibility of the protein-bound metal ions to small donor molecules in solution. From these experiments, upper and lower limits can be established for the distance of each transferrin-bound metal ion from the surface of the protein.

All mechanisms of radiationless energy transfer are dependent on the separation between the donor and acceptor. Many investigators have utilized fluorescence energy transfer via the Förster dipole–dipole mechanism to determine distances between *fixed* donors and acceptors [see, for example, the review by Stryer (1978)]. Other workers have studied the case where donor and acceptor diffuse independently, such that the distance between donors and acceptors changes significantly during the donor lifetime (Galanin, 1960; Steinberg & Katchalski, 1968; Thomas et al., 1978). The "rapid diffusion limit", beyond which any additional increase in diffusion coefficients does not further enhance the rate of energy transfer, is reached when $(6D\tau)^{1/2} \gg s$ (where D = the sum of diffusion coefficients of donor and acceptor, τ = lifetime of the donor, and s = the average separation between acceptors). In the rapid diffusion limit, each energy donor is influenced by many possible acceptors during its lifetime, and the rate of energy transfer will depend on the freedom of access of donors to acceptors (which may be bound to macromolecules). A long-lived donor, such as $^5D_4$ terbium with its millisecond lifetime, is necessary to obtain this diffusion-enhanced energy transfer. The emission of terbium occurs in the visible region (principally at 546 nm), and therefore compounds which absorb visible light are suitable energy acceptors.

Accordingly, an electrically neutral terbium chelate, freely diffusing in solution, can be used to probe the iron-binding sites of transferrin. The more accessible an iron chromophore is, the larger the second-order rate constant for energy transfer will be. In addition, the second-order rate constant can be interpreted in terms of an "apparent distance of closest approach" between donor and acceptor, which provides quantitative information on the geometry of each metal-binding site.

Here we describe energy transfer experiments (in the rapid diffusion limit) between metal chelates and metallotransferrins in which the donor and acceptor have been varied by changing chelator, metal ion, or anion.

Experimental Procedures

All reagents were the purest commercially available and used without further purification. L-1-Bzl-EDTA[1] was synthesized as described by Yeh et al. (1979). Human serum transferrin (iron-free, 99% electrophoretically pure) was purchased from Calbiochem-Behring. All water used for buffers was deionized and distilled; all labware was acid-rinsed (Thiers, 1957).

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[1] Abbreviations used: Tb–HED3A, the complex formed by terbium(III) and *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; $Fe(NTA)_2$, the complex formed by ferric ion and nitrilotriacetate in a 1:2 molar ratio; Tb–Bzl-EDTA, the complex formed by terbium(III) and L-1-benzylethylenediaminetetraacetic acid; Tf, human serum transferrin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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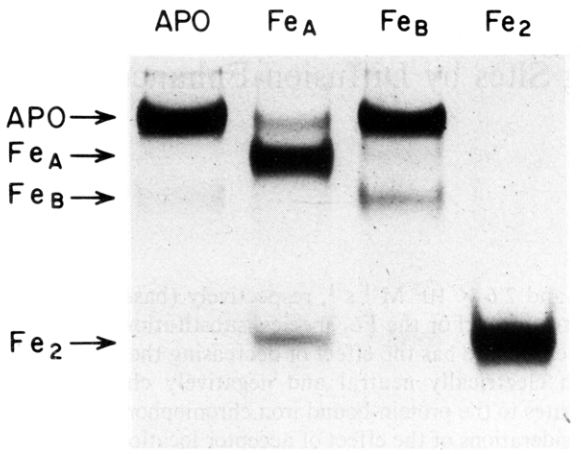

FIGURE 1: Analysis of the composition of iron–transferrin samples by polyacrylamide gel electrophoresis in 6 M urea (Makey & Seal, 1976). Arrows along the left side indicate positions of the various species after electrophoresis. Labels along the top indicate the component of interest in each sample. Iron-free transferrin (which is not an energy acceptor) is denoted APO; monoferric transferrin with iron at the "A" site is denoted Fe_A; monoferric transferrin with iron at the "B" site, Fe_B; diferric transferrin, Fe_2. Recently, Frieden & Aisen (1980) have proposed that Fe_A–transferrin be denoted $TfFe_C$ because the metal ion is bound near the protein C terminus and that Fe_B–transferrin be denoted Fe_NTf because the metal ion is bound near the protein N terminus.

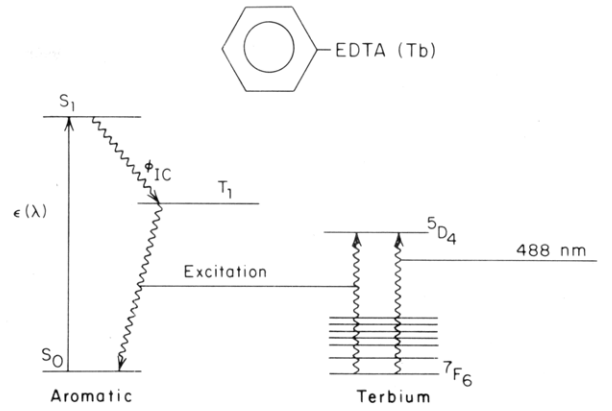

FIGURE 2: The two pathways used for excitation of terbium in Tb–Bzl-EDTA to the luminescent $^5D_4$ state. Absorption of 488-nm light by terbium ($\epsilon_{488} \simeq 0.05$ M^{-1} cm^{-1}) causes the direct transition from the ground $^7F_6$ level to the $^5D_4$ level. Alternatively, absorption of ultraviolet radiation by the aromatic ring ($\epsilon_{257} \gtrsim 10^3$ M^{-1} cm^{-1}) can cause the $S_0 \rightarrow S_1$ transition in that group, which may be followed by singlet–triplet intersystem crossing to the T_1 level; finally, transfer of energy from the aromatic T_1 level to the adjacent terbium ion can be facilitated by overlap of electron clouds.

$FeCl_3$ solutions were prepared from iron wire dissolved in 6 M HCl; millimolar stock solutions of iron oxalate (4 mol of oxalate/mol of iron) and iron nitrilotriacetate [$Fe(NTA)_2$] were prepared by addition of $FeCl_3$ solution to the dissolved ligand and careful adjustment with NaOH to a final pH of 3.5–4.0. The resulting solutions were standardized with ferrozine (Harris & Aisen, 1975).

Metallotransferrins. Millimolar apotransferrin solutions were made up in the following buffers: 25 mM HCO_3^-, pH 8.3, and 25 mM HCO_3^-–10 mM Hepes, pH 7.4, for the bicarbonate forms of iron transferrin; degassed 10 mM Hepes, pH 7.4, for the oxalate form of iron transferrin; 0.1 M Tris–10 mM HCO_3^-, pH 8.9, for dimanganic transferrin. Titrations with $Fe(NTA)_2$ (monitored at 465 nm) were performed on apoprotein samples to determine available metal-binding sites. In all cases, over 90% of the binding sites were available, based on quantitation of apoprotein by absorbance at 280 nm (Harris & Aisen, 1975).

The three (bi)carbonate forms of iron transferrin were made by addition of stoichiometric amounts of $Fe(NTA)_2$ for diferric transferrin and for monoferric transferrin with the iron bound at site "A" (Fe_A form) and by addition of stoichiometric amounts of $Fe(NH_4)_2(SO_4)_2$ for the monoferric Fe_B form (Schlabach & Bates, 1975; Aisen et al., 1978; Evans & Williams, 1978). Frieden & Aisen (1980) have recently proposed that Fe_A–transferrin be denoted $TfFe_C$ because the metal-binding site occupied is near the protein C terminus and that Fe_B–transferrin be denoted Fe_NTf because the bound metal is near the N terminus.

Samples were either dialyzed for 48 h (four changes of 250 volumes each) against the desired buffer or applied to Sephadex G-25 columns to remove nitrilotriacetate or low molecular weight impurities. Urea (6 M)–polyacrylamide gels were run on a Bio-Rad 60 × 135 mm slab gel electrophoresis unit to check the purity of Fe_A, Fe_B, or diferric transferrin preparations (Makey & Seal, 1976; Aisen et al., 1978); after staining a gel with Coomassie Blue and destaining, a Joyce-Loebl MK III C double-beam microdensitometer was used to determine the proportion of each form of transferrin in a given sample. A typical gel is shown in Figure 1. The oxalate form of diferric transferrin was prepared by addition of a stoichiometric amount of iron oxalate (Schlabach & Bates, 1975). All concentrations were checked by visible absorbance measurements at 465 nm (Schlabach & Bates, 1975). Diterbium transferrin was prepared by adding an excess of Tb(III) from a stock solution of pH 3.5 $TbCl_3$ solution. Dimanganic transferrin was prepared by oxidation of Mn(II) in solution with apotransferrin in 0.1 M Tris–10 mM HCO_3^-, pH 8.9, overnight [similar to the O_2 oxidation method of Inman (1956) except under positive pressure of O_2]. For experiments, the buffer was changed to either 25 mM HCO_3^-, pH 8.3, or 25 mM HCO_3^-–10 mM Hepes, pH 7.4, by dialysis or gel filtration as described for purification of (bi)carbonate iron transferrins. Formation of manganic transferrin was monitored by visible absorbance measurements at 429 nm ($\epsilon_{429} = 8.72 \times 10^3$ M^{-1} cm^{-1}) (Inman, 1956; Tomimatsu et al., 1976; Aisen et al., 1969).

Metal Chelates. Terbium chelates used as energy donors were prepared by addition of millimolar $TbCl_3$ solution to aqueous solutions of EDTA, HED3A, and L-1-Bzl-EDTA, allowing a 20% excess of chelating agent. Hexadentate Co(III)–Bzl-EDTA was prepared by H_2O_2 oxidation of the Co(II) chelate (Shimi & Higginson, 1958); the compound was purified by Bio-Rad AG-1 (acetate) anion-exchange chromatography with a 0–1.5 M NH_4OAc gradient.

Electronic Transitions of Terbium. As shown in Figure 2, the lowest radiative level, $^5D_4$, of a bound terbium ion can be populated indirectly by UV excitation of an adjacent aromatic group (Crosby et al., 1962), such as a coordinated tyrosinate side chain of transferrin (Feeney & Komatsu, 1966; Luk, 1971) or the benzyl group in the case of terbium–Bzl-EDTA (Yeh et al., 1979). Alternatively, the $^5D_4$ state may be populated directly by excitation of the bound terbium ion with 488-nm light from an argon ion laser. Since the extinction coefficient of an aromatic ring in the UV ($\epsilon \approx 10^3$ M^{-1} cm^{-1}) is far greater than that of terbium at 488 nm ($\epsilon \simeq 0.05$ M^{-1} cm^{-1}), an ordinary xenon lamp would be suitable for UV excitation, while the input intensity of a laser is required to directly excite terbium at 488 nm.

As shown in Figure 3, pathways for deexcitation of Tb include luminescence (k_f), energy transfer to a chromophoric acceptor ($k_T = k_2[A]$), or nonradiative energy loss to O–H

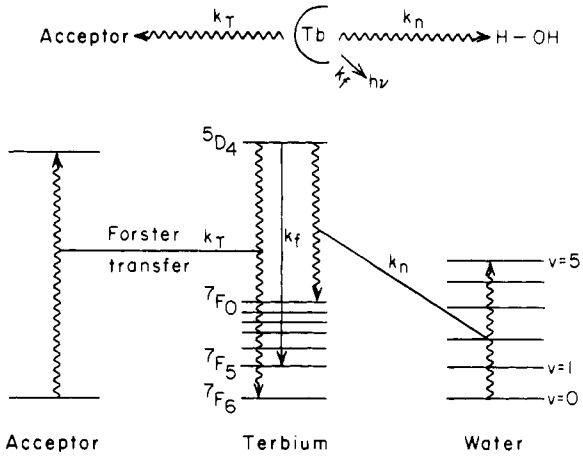

FIGURE 3: Major pathways for deexcitation of luminescent $^5D_4$ terbium include energy transfer to a chromophoric acceptor ($k_T = k_2[A]$), photon emission (k_f), and nonradiative loss of energy to coordinated water (k_n).

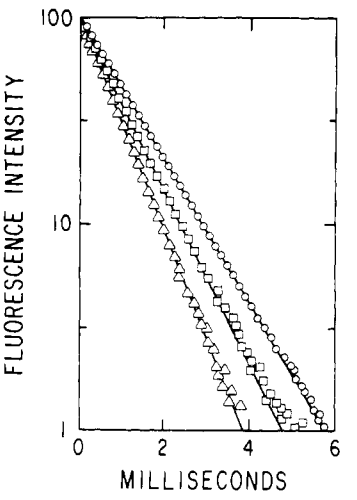

FIGURE 4: Typical semilogarithmic plots of fluorescence intensity vs. time for Tb–HED3A in the presence of 1.2 mM apotransferrin (○), 1.0 mM (monoferric) Fe_A–transferrin–(bi)carbonate (□), or 0.7 mM Fe_2–transferrin–(bi)carbonate (△) in 25 mM $NaHCO_3$, pH 8.3, 298 K.

oscillators (e.g., water molecules) in the first coordination sphere of the terbium ion (k_n). The energy gap between the $^5D_4$ and $^7F_0$ levels of terbium approximately matches the energy of five vibrational quanta of the O–H group (Stein & Würzberg, 1975). It is important to note that for the terbium chelates used in these studies, approximately three water molecules are directly coordinated to each chelated terbium ion. Displacement of a water molecule by another ligand will be revealed experimentally by a large increase in the fluorescence lifetime of the terbium. For example, negatively charged metal chelates such as Fe(III)–EDTA bind weakly to unoccupied metal-binding sites of transferrin (Bates et al., 1967; Rogers et al., 1977); binding of anionic Tb(III)–EDTA to apotransferrin is readily observable by terbium lifetime measurements because the presence of millimolar apotransferrin lengthens the terbium lifetime from 1.26 to 1.44 ms (presumably by displacing coordinated H_2O). Horrocks & Sudnick (1979) have considered in detail the effect of H_2O coordinated to terbium.

Quantum Yield. This was determined by using a Perkin-Elmer MPF-2A fluorescence spectrophotometer, according to the procedures of Weber & Teale (1957). The integrated fluorescence emission from Tb–EDTA in aqueous solution was compared to that from eosin Y in 0.1 N NaOH ($Q = 0.19$) by using 488-nm light for excitation. The quantum yield for aqueous Tb–EDTA was found to be $Q_0 = 0.2 \pm 0.05$ at room temperature. The alcohol OH group of Tb–HED3A makes its quantum yield slightly smaller, but still within experimental error of 0.2.

Terbium Fluorescence Lifetimes. The apparatus used for fluorescence lifetime measurements is described elsewhere (Thomas et al., 1978). Because the protein efficiently "filters" ultraviolet light, samples were irradiated by the 488-nm argon laser line in most of the experiments reported here; ultraviolet excitation used 257-nm radiation obtained by frequency doubling the 514-nm laser emission. After pulsed excitation, terbium fluorescence intensity at 546 nm was monitored as a function of time. Practically perfect single-exponential decays were obtained, from which lifetimes were computed by the method of Thomas et al. (1978). Typical data for the fluorescence decay of Tb–HED3A in the presence of transferrin are shown in Figure 4.

Each sample was prepared by adding a solution of metal chelate (≤20 μL) to 100 or 200 μL of protein solution immediately before measurement of the lifetime. Typically, protein concentrations were 1 mM and chelate concentrations ranged from 0.2 to 2 mM. After lifetime measurements, acceptor concentrations in the experimental samples were rechecked by absorbance measurements at 465 nm for Fe(III)–transferrins, at 429 nm for Mn(III)–transferrin, and at 538 nm for Co(III)–Bzl-EDTA. Controls for each experiment were the Tb chelate in buffer and the Tb chelate plus apotransferrin in buffer.

In all calculations the value of τ_0, the lifetime of excited terbium in the absence of chromophoric energy acceptors, was obtained from the terbium chelate in buffer. For Tb–EDTA or Tb–Bzl-EDTA in H_2O at neutral pH, $\tau_0 = 1.17$ ms; for Tb–HED3A in H_2O at neutral pH, $\tau_0 = 1.03$ ms; for Tb_2–transferrin–(bi)carbonate at pH 7.4–8.3, $\tau_0 = 1.25$ ms. For the small Tb–HED3A chelate in oxalate, bicarbonate, or Hepes buffers at pH 7.4–8.3, τ_0 was as long as 1.27 ms, 23% higher than its value in H_2O alone. This may indicate a weak association with anions in the buffer, which would cause the average terbium complex to have a fractional negative charge. The results of these experiments must be interpreted with this possibility in mind. However, for Tb–HED3A, τ_0 measured in buffer alone was within 4% of τ_0 measured in buffer containing apotransferrin; therefore, Tb–HED3A—in contrast to uninegative Tb–EDTA—does not bind significantly to unoccupied transferrin metal-binding sites. In donor–acceptor experiments, τ values ranged from 40% to 85% of τ_0. Lifetime measurements were reproducible in duplicate samples to within a maximum error of 1%.

Results

In the rapid diffusion limit, the average distance s by which acceptors are separated must be much less than $(6D\tau)^{1/2}$, where D is the relative diffusion coefficient of donor and acceptor and τ is the lifetime of excited donor. According to Chandrasekhar (1943), $s = 0.554/n^{1/3}$, where n = density of acceptors (molecules m^{-3}); since our typical transferrin acceptor concentration is 10^{-3} M, $s \approx 10^{-8}$ m. Because $D \approx 10^{-9}$ m^2 s^{-1} for a small molecule in solution relative to a protein and $\tau \approx 10^{-3}$ s, the relative distance diffused by a donor during its lifetime is $(6D\tau)^{1/2} \geq 10^{-6}$ m. Since $(6D\tau)^{1/2}$ is more than 2 orders of magnitude greater than s, the average donor will encounter many acceptors during its millisecond lifetime. As a result of this, all donors sample the same (quasi-equilibrium) distribution of acceptors in solution about them. Therefore,

Table I: Apparent Distances of Closest Approach (in Nanometers) between Freely Diffusing Donors and Acceptors[a]

	acceptor					
donor	Fe_A-Tf-HCO_3	Fe_B-Tf-HCO_3	Fe_2-Tf-$(HCO_3)_2$	Fe_2-Tf-$(C_2O_4)_2$	Mn_2-Tf-$(HCO_3)_2$	Co(III)-Bzl-EDTA
Tb-HED3A	2.09 ± 0.15	1.81 ± 0.10	1.46 ± 0.04	1.80 ± 0.06	1.44 ± 0.05	
Tb-EDTA			1.54 ± 0.05	2.02 ± 0.07		
Tb-Bzl-EDTA			1.27 ± 0.04		1.42 ± 0.05	
Tb_2-Tf-$(HCO_3)_2$			*b*			1.5 ± 0.1
R_0 (nm)	2.71	2.71	2.71	2.96	3.04	2.12

[a] Calculated from eq 5 by using experimentally measured second-order rate constants, $\tau_0 = 1.24$ ms, and R_0 as given in the table for the various donor-acceptor combinations. [b] Too large to measure.

single-exponential decay in fluorescence intensity is observed (Figure 4), from which the lifetime of freely diffusing terbium chelates in the presence of transferrins may be accurately determined.

The rate of donor decay is given by

$$-\frac{d[Tb^*]}{dt} = \frac{1}{\tau}[Tb^*] = \left(\frac{1}{\tau_0} + k_2[A]\right)[Tb^*] \quad (1)$$

where [Tb*] is the molar concentration of excited donor, τ is the lifetime of excited donor, τ_0 is the lifetime of excited donor in the absence of acceptor, k_2 is the second-order energy transfer rate constant, and [A] is the concentration of acceptor.

In a mixture containing several different acceptors, a term like $k_2[A]$ must be included in eq 1 for each acceptor. For example, pure Fe_2-transferrin-(bi)carbonate may be prepared (Figure 1), and its energy-transfer rate constant determined from donor lifetime measurements according to eq 2a. But

$$k_2 = \frac{1}{2[Fe_2Tf]}\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right) \quad (2a)$$

in preparation of Fe_A-transferrin-(bi)carbonate, some Fe_2-transferrin-(bi)carbonate is also formed, as indicated in the Fe_A sample of Figure 1. From a densitometer scan of the gel in Figure 1, the relative concentrations of each transferrin species in a given sample may be determined, and the rate constant for energy transfer to Fe_A-transferrin-(bi)carbonate may be calculated from eq 2b. Note that apotransferrin is

$$k_A = \frac{1}{[Fe_ATf]}\left(\frac{1}{\tau} - \frac{1}{\tau_0} - 2k_2[Fe_2Tf]\right) \quad (2b)$$

not an energy acceptor.

Finally, the Fe_B-transferrin-(bi)carbonate sample contains small amounts of the Fe_2 and the Fe_A species, so that its rate constant may be calculated from eq 2c.

$$k_B = \frac{1}{[Fe_BTf]}\left(\frac{1}{\tau} - \frac{1}{\tau_0} - 2k_2[Fe_2Tf] - k_A[Fe_ATf]\right) \quad (2c)$$

An important parameter in dipolar energy transfer is R_0, the distance at which transfer efficiency is 50%. R_0 is defined as (Stryer, 1978)

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6}(9.79 \times 10^2)\text{nm} \quad (3)$$

where

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

For metal-metal energy transfer κ^2, the orientation factor between donor and acceptor, is taken to be $^2/_3$ because metal ions are practically unpolarized donors and acceptors (Horrocks et al., 1975). The quantum yield of the donor in the absence of acceptor is $Q_0 = 0.2$. The index of refraction of the solution is $n = 1.33$. The spectral overlap integral, J, can be calculated from the donor's emission spectrum and the acceptor's absorption spectrum (Thomas et al., 1978). $F(\lambda)$ is the fraction of donor emission at wavelength λ, and $\epsilon(\lambda)$ is the extinction coefficient of the acceptor at wavelength λ, in units of M^{-1} cm^{-1}. The absorption spectra taken for dimanganic transferrin-(bi)carbonate were in good agreement with those of Tomimatsu et al. (1976) and Aisen et al. (1969). R_0 calculated for each donor-acceptor pair is given in Table I.

Discussion

Radiationless energy transfer may occur by several different mechanisms (Dexter, 1953), all of which depend strongly on the distance between donor and acceptor. The energy-transfer mechanism with the weakest distance dependence, which therefore acts at the longest distances, is the dipole-dipole interaction studied by Förster (1948). In the rapid diffusion limit, where each donor molecule encounters many possible acceptors during its lifetime, energy transfer depends on the *distance of closest approach* between donor and acceptor (Galanin, 1960; Thomas et al., 1978). If the steric properties of these molecules prevent close donor-acceptor contact, then only the dipole-dipole mechanism can contribute significantly to energy transfer. This leads to the following experimental criterion for assessing the mechanism by which energy is transferred from a small metal chelate to a protein-bound acceptor: if the observed second-order rate constant is much smaller than the value *calculated* for a purely dipole-dipole process between two *small* molecules, then the distance of closest approach is likely to be large and the dipole-dipole mechanism dominant. Conversely, the experimental observation of unexpectedly *large* rate constants for energy transfer suggests that direct contact between donor and acceptor is possible and that other mechanisms—particularly the exchange mechanism (Dexter, 1953; Meares et al., unpublished experiments)—are important.

For dipole-dipole energy transfer between *spherical* donors and acceptors in the rapid diffusion limit, the second-order rate constant is given by eq 5, with all distances in nanometers,

$$k_2 = \frac{0.602R_0^6}{\tau_0}\int_a^\infty \frac{4\pi r^2\, dr}{r^6} = \frac{2.523R_0^6}{\tau_0 a^3}\ M^{-1}\ s^{-1} \quad (5)$$

where a is the distance of closest approach between donor and acceptor. (If the molecules involved are not spheres, then the lower limit of the integral in eq 5 will depend on the relative orientation of donor and acceptor, and angular variables must be considered explicitly.) Comparison of calculations based on eq 5 with experimentally observed second-order energy transfer rate constants may be used to rule out the possibility that either ferric ion is bound on the surface of transferrin.

Using $R_0 = 2.71$ nm, $\tau_0 = 1.24 \times 10^{-3}$ s, and $a = 0.8$ nm in eq 5, we calculate a second-order rate constant of 1.6×10^6 M^{-1} s^{-1}. The magnitude of this rate constant represents what would be expected for dipole–dipole energy transfer between electrically neutral Tb–HED3A (radius 0.4 nm) and a "small" transferrin iron chromophore about the size of an EDTA chelate (radius 0.4 nm), hypothetically excised from the protein. The experimental second-order rate constants for all iron transferrin species are in fact 1 order of magnitude *smaller* than this [e.g., 0.9×10^5 M^{-1} s^{-1}, 1.4×10^5 M^{-1} s^{-1}, and 2.6×10^5 M^{-1} s^{-1} for Fe_A–, Fe_B–, and Fe_2–transferrin–(bi)-carbonate, respectively, based on iron concentration]. This suggests that the iron chromophores are buried beneath the protein's surface and therefore that only the dipole–dipole mechanism of energy transfer need be considered [see Dexter (1953)].

Further support for the dipole–dipole mechanism is provided by the comparison of rate constants for energy transfer from Tb–HED3A to Mn_2–transferrin–(bi)carbonate ($k_2 = 5.3 \times 10^5$ M^{-1} s^{-1}) and Fe_2–transferrin–(bi)carbonate ($k_2 = 2.6 \times 10^5$ M^{-1} s^{-1}). The ratio of these rate constants is 2.04. If the two metallotransferrins have the same shape, the dipole–dipole interaction would lead to a ratio of rate constants just equal to the ratio of values of R_0^6; from Table I this is $(3.04/2.71)^6 = 1.99$. Conversely, this agreement might be taken as evidence that changing the metal ion from Fe(III) to Mn(III) does not change the shape of the metalloprotein.

For metallotransferrins, it is instructive to use experimentally determined values of k_2 in eq 5 to calculate *apparent* distances of closest approach between the small metal chelates and the protein-bound metal ions. These numbers are given in Table I. For an electrically neutral donor, the apparent distance of closest approach provides a measure of the *maximum* distance that a chromophore can be buried in a macromolecule. If a spherical donor with radius R_D is used in the experiment (such as Tb–HED3A, $R_D = 0.4$ nm), then the acceptor chromophore must be within $a - R_D$ of the surface in at least one direction. To see this, imagine a sphere with radius $a - R_D$, centered on the acceptor chromophore; if the donor were sterically prevented (by protein) from ever touching the surface of this sphere, then the distance of closest approach would have to be greater than a by definition. Therefore, the acceptor must be *no more than* $a - R_D$ away from the nearest surface of the protein, but it would be exactly that distance away if it were in the center of a sphere with radius $a - R_D$. Thus, the values given in the first row of Table I represent experimentally measured upper bounds to the minimum possible separation between Tb–HED3A and the acceptors studied. For the other metal chelates, the situation may be complicated by electrostatic effects, but these are expected to be small (Meares et al., unpublished experiments).

Nuclear magnetic relaxation dispersion, electron spin resonance, and visible absorption measurements have demonstrated the accessibility of the iron-binding sites to water and to bulky auxiliary anions, thus implying that the bound ferric ions are located close to the protein surface (Koenig & Schillinger, 1969a,b; Harris et al., 1979; Schlabach & Bates, 1975). Comparison of the apparent distance of closest approach of Tb–HED3A to Fe_2–transferrin–(bi)carbonate ($a = 1.46 \pm 0.04$ nm) and to Fe_2–transferrin–oxalate ($a = 1.80 \pm 0.06$ nm) suggests that the metal lies near the surface of the protein, because changing the anion has such a large effect on the apparent distance of closest approach. That is, the acceptor-centered spherical model of eq 5 appears to be a poor approximation of the true geometry because, if the acceptor were buried in the center of a sphere of radius $a - R_D$, the effect of the anion substitution could not measurably alter a without grossly changing the conformation of the protein. On the other hand, since a major portion of the observed energy transfer will occur when the donor is closest to the acceptor, placement of the acceptor near the surface of the protein would make energy transfer more sensitive to small conformational changes at the acceptor site. The observation that the ferric acceptor becomes *less* accessible when the (bi)carbonate ion is replaced by the larger oxalate ion is consistent with the possibility that the anion lies between the bound metal ion and the solution.

The energy-transfer experiments with monoferric transferrins show that the iron-binding sites in Fe_2–transferrin–(bi)carbonate are more accessible than in either monoferric form (see Table I). The apparent distance of closest approach to binding sites on diferric transferrin is a weighted average of the distance to the "A" and "B" sites. If no conformational changes occurred, we would expect the apparent distance of closest approach of Tb–HED3A to Fe_2–transferrin–(bi)-carbonate to be 1.93 nm, i.e., $1/a_{Fe_2}^3 = (1/2)(1/a_{Fe_A}^3 + 1/a_{Fe_B}^3)$. However, the experimentally observed distance is 1.46 nm. This shorter experimental distance is consistent with the protein becoming more compact as monoferric transferrin binds a second ferric ion (Rosseneu-Motreff et al., 1971). The physical reason for this is that decreased exclusion of the terbium probe from any region near the protein-bound Fe(III) will decrease the apparent distance of closest approach.

Taken together, the experiments in the first row of Table I imply that the metal-binding sites of transferrin are (1) not exposed on the protein's surface, (2) no more than 1.7 nm below the surface (2.09 – 0.4 nm), and (3) probably separated from the surface by less than 1.7 nm.

Results with electrically neutral Tb–HED3A and uninegative Tb–EDTA as donors suggest possibilities about the electric charge distribution around the binding sites. For Fe_2–transferrin–(bi)carbonate, a for the two different donors is the same within experimental error (Table I). It would thus appear that when (bi)carbonate is the auxiliary anion, the regions around the two iron chromophores have little or no net electric charge. However, for Fe_2–transferrin–oxalate, there is a small but significant difference between the values of a for Tb–EDTA and Tb–HED3A. This difference suggests that the regions surrounding the two iron chromophores have a detectable net negative charge, which repels uninegative Tb–EDTA but not electrically neutral Tb–HED3A. While no definitive statement can be made, the combined results are consistent with the possibility that the physiological anion is the uninegative HCO_3^-, rather than dinegative CO_3^{2-} [however, see Rogers et al. (1977)].

Because the equations for energy transfer are unaltered if donor and acceptor are interchanged, study of energy transfer from protein-bound terbium to a freely diffusing transition metal chelate is also interesting. The apparent distance of closest approach of Co(III)–Bzl-EDTA to diterbium transferrin was 1.5 nm, which is consistent with the other experimental data. Furthermore, when diterbium transferrin was added to pure diferric transferrin, no energy transfer was observed—in accord with the expectation that close approach between donor and acceptor would not be possible in this case. While not further developed here, such "reversed" experiments may be useful in studying other metal-binding macromolecules such as calcium-binding proteins [Tb(III) is the same size as Ca^{2+}]. This approach has the virtue that R_0 may be varied easily by using different acceptors but the disadvantage that

for most proteins it is almost impossible to accurately determine the quantum yield Q_0 of a bound lanthanide ion because of limited solubility (Horrocks et al., 1975).

Diffusion-enhanced energy transfer can be used in a variety of ways to provide information about diffusion rates, electrostatic properties, and geometric properties of chromophores in biological systems (Thomas et al., 1978; Meares et al., unpublished experiments; Meares & Rice, 1980). While individual experimental results should be interpreted conservatively until a large body of data is analyzed, it is clear that this technique provides a fundamentally important new way of looking at macromolecules in solution.

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