Energy Transfer between Terbium and Iron Bound to Transferrin: Reinvestigation of the Distance between Metal-Binding Sites†

Claude F. Meares* and Joseph E. Ledbetter

ABSTRACT: The addition of trivalent iron, gallium, and terbium ions to the metal binding sites of human transferrin has been investigated by fluorescence and spectrophotometric measurements. Results are consistent with the possibility that the addition of ferric nitrilotriacetate to apotransferrin does not lead to a random distribution of iron bound to the two metal-binding sites on the protein, but rather an asymmetric distribution with iron bound mainly to one of the sites. The subsequent addition of terbium leads to the binding of terbium ions to the vacant sites on monoferric transferrin molecules, and observations of the intensity of terbium fluorescence from such samples provides clear evidence of transfer of excitation energy from the terbium site to the iron site. These results lead to the estimate that the two metal-binding sites of human transferrin are separated by a distance of 25 ± 2 Å, in disagreement with an earlier report (Luk, C. K. (1971), *Biochemistry 10,* 2838) that the sites were separated by more than 43 Å. Consideration of the dimensions of transferrin indicates that the two sites lie relatively close to each other on the macromolecule.

Human transferrin, the iron-transport protein of blood plasma, possesses two specific metal-binding sites which appear to be separated by at least 9 Å (Aasa et al., 1963; Windle et al., 1963). These sites, which may have different physiologic functions (Fletcher and Huehns, 1967), are capable of binding a variety of metal ions, including Fe(III), Ga(III), and Tb(III) (Holmberg and Laurell, 1945; Harris et al., 1974; Luk, 1971). It has been shown in all cases investigated (including iron and gallium) that the binding of each metal ion must be accompanied by the binding of an anion such as carbonate (or bicarbonate) to the protein.

An attempt to obtain further information about the spatial relationship between the metal-binding sites has been reported by Luk (1971). In the course of his study on the binding of trivalent lanthanide ions to human transferrin, it was shown that terbium ions become highly fluorescent upon binding to the protein. It was reported that the fluorescence intensity of transferrin-bound terbium(III) was insensitive to the addition of Eu(III), Ho(III), Er(III), Fe(III), or Cu(II), leading to the conclusion that the two metal-binding sites of transferrin were separated by a large distance compared with the critical distance for nonradiative transfer of electronic excitation energy from a terbium "donor" to another metal "acceptor" (Förster, 1948; Dexter, 1953). It was estimated that the values of this critical distance, R_0, for the various donor–acceptor pairs varied from about 10 Å for lanthanide acceptors to 27 Å for Fe(III), and a lower limit on the separation between the metal-binding sites was estimated to be 43 Å (Luk, 1971).

Subsequent to that report, considerable progress has been made in understanding the chemistry involved in addition of metal ions to transferrin; in particular, Bates and Schlabach (1973) have shown that addition of simple ferric salts to apotransferrin (the method apparently used by Luk) results in low yields and complicated side reactions, while the addition of the nitrilotriacetate chelate of Fe(III) results in rapid, quantitative, specific addition of ferric ions to the metal-binding sites on transferrin. Taking advantage of such technical advances, we have investigated the preparation and fluorescence properties of solutions of transferrin molecules containing terbium ions bound to the available metal-binding sites of monoferric transferrin, monogallic transferrin, and apotransferrin. Comparison of terbium fluorescence from these solutions provides new information on the separation between the metal-binding sites.

Experimental Procedure

Precautions were taken to avoid contamination by extraneous metal ions. Labware was soaked in mixed acids (Thiers, 1957) and H_2O was deionized, filtered, and distilled before use. Buffers were chosen for their lack of contamination by heavy metals. Iron-free human transferrin (electrophoretic purity 98–99%) was obtained from Behring Diagnostics. It was characterized by titration with standard ferric nitrilotriacetate solution (Bates and Schlabach, 1973; Harris and Aisen, 1975). Some samples were rendered free of possible contaminating metal ions by dialysis for 4 h against 10 mM EDTA,[1] pH 4.6, then for 6 h against 0.1 M $NaClO_4$, and then for 20 h against 10 changes of distilled, deionized H_2O, followed by lyophilization.

Standard 4 mM ferric nitrilotriacetate solutions were prepared from iron wire and calibrated using ferrozine (Harris and Aisen, 1975). Standard solutions of gallium nitrilotriacetate or gallium nitrate were prepared from a standard 1000 ppm gallium solution (Ventron). Standard terbium chloride solutions were prepared from $TbCl_3 \cdot 6H_2O$ (Ventron). Spectrophotometric titrations against identical samples of apotransferrin were used to check the concentrations of the three metal solutions. Measurements were made using Cary Model 17 and Gilford Model 250 spectrophotometers. Titrations with gallium were monitored at 242 nm; with terbium, 295 nm; and with iron, 470 nm. Consistent end points of 2 metal ions per protein molecule, reproducible to within 5%, were obtained in all cases.

Samples of transferrin partially saturated with iron were prepared by combining known amounts of standard ferric

† From the Department of Chemistry, University of California, Davis, California 95616. *Received May 10, 1977.* This work was supported in part by National Institutes of Health Grants CA 16861 and HL 18619.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SD, standard deviation.

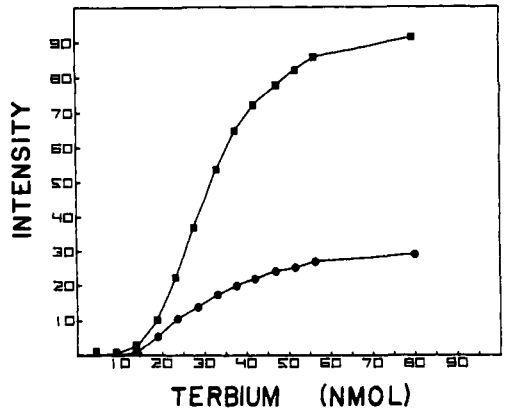

FIGURE 1: Comparison of the intensity (in arbitrary units) of the terbium emission line at 546 nm as a function of the addition of Tb(III) to 7 nmol of metal-binding sites on apotransferrin (■) or on monoferric transferrin (●) in 3.0 mL of 25 mM $NaHCO_3$, 14 μM nitrilotriacetate, pH 8.3. Excitation wavelength is 288 nm.

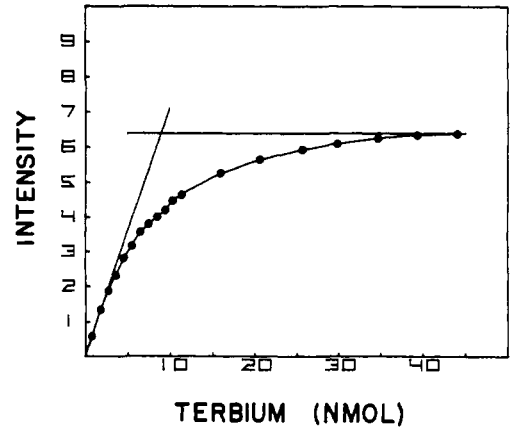

FIGURE 2: Fluorescence titration of 3.5 nmol of apotransferrin (7 nmol metal-binding sites) with terbium. Fluorescence intensity is in arbitrary units, excitation wavelength is 288 nm, and emission wavelength is 546 nm. The sample was dissolved in 3.0 mL of 25 mM $NaHCO_3$, pH 8.3.

nitrilotriacetate solution with apotransferrin. It has recently been shown by Harris (1977) that, over a range of pH, transferrin binds iron preferentially at one of its two metal-binding sites. With the aim of leaving very little apotransferrin in solution, we added iron up to 75% of saturation; in this case, even if iron is bound in a random fashion, 75% of the unoccupied metal-binding sites will lie on monoferric transferrin molecules. It is noteworthy that similar fluorescence quenching results were obtained over a range of iron saturation from 45% to 75%. This suggests that iron is bound preferentially to one of the sites and is consistent with the results of Harris (1977).

Fluorescence Measurements. The protein was dissolved in either 5 mM $NaHCO_3$, 30 mM Hepes, pH 8.2, or in 25 mM $NaHCO_3$, pH 8.3. Transferrin solutions were passed through 0.22-μm filters before characterization in order to remove traces of debris which might interfere with fluorescence measurements, and only freshly prepared solutions were used for these experiments. Appropriate amounts of metal ions were added to aliquots of 10^{-4} M apotransferrin and incubated for at least 30 min at room temperature before dilution and fluorescence measurement. Under these conditions, equilibrium is reached within a few minutes. The concentration of nitrilotriacetate was carefully controlled, and the order of addition of the metals was varied in order to assure that the same endpoint was always reached. Measurements of terbium fluorescence were made at room temperature with a Perkin-Elmer MPF-2A fluorescence spectrophotometer operated in the ratio mode. In the range of concentration between 0.3 μM and 3 μM, fluorescence intensity was found to be directly proportional to concentration. In this report comparisons are made only between solutions having identical concentrations of terbium, nitrilotriacetate, and available transferrin metal-binding sites.

Results

In Figure 1, a terbium fluorescence titration of 7 nmol of apotransferrin metal-binding sites (in 3.0 mL of 25 mM $NaHCO_3$, 14 μM nitrilotriacetate) is compared with a terbium fluorescence titration of 7 nmol of monoferric transferrin metal-binding sites (in the same buffer). It was demonstrated by appropriate control experiments that the presence of nitrilotriacetate in the buffer leads to the formation of the nonfluorescent terbium nitrilotriacetate chelate and thereby depresses formation of the highly fluorescent terbium–transferrin complex during the initial stage of the titration. After this initial stage, the ratio of fluorescence intensities rapidly reaches the value 3.15 ± 0.07 (SD), which is maintained even after the addition of a large excess of terbium. After correction for the absorbance of the samples, the ratio has the value 2.8. At the end of each titration, the addition of a stoichiometric amount of ferric nitrilotriacetate rapidly reduces the terbium fluorescence intensity to zero; this suggests, but does not prove, that terbium binds only to the two known metal-binding sites on the protein. The shapes of the terbium excitation and emission spectra were identical for monoterbium monoferric transferrin, diterbium transferrin, and monoterbium transferrin, implying that Förster's (1948, 1965) theory of excitation transfer for the case of "very weak coupling" is applicable.

The possibility that the difference in terbium fluorescence intensities shown in Figure 1 is due to an intrinsic difference between transferrin metal-binding sites was investigated by fluorescence titration of apotransferrin with terbium in the absence of nitrilotriacetate. Intrinsic differences in the fluorescence properties of the two sites should be revealed by comparison of the fluorescence intensity per mole of bound terbium at the beginning and at the end of the titration. As shown in Figure 2, the initial slope of the titration curve has the value 0.71 div/nmol of Tb added. From plots of the reciprocal of fluorescence intensity against the reciprocal of free terbium concentration, it was found that, in 25 mM $NaHCO_3$, terbium binds to the sites on transferrin with different apparent dissociation constants, having values $K_1 \cong 4 \times 10^{-7}$ and (roughly) $K_2 \approx 4 \times 10^{-5}$. The value of K_1 indicates that, in the experiment of Figure 2, 71% of initially added terbium becomes bound to the protein, so the initial slope has the value 1.0 div/nmol of Tb bound. Therefore, if the two sites have identical fluorescence properties, the fluorescence intensity should asymptotically approach 7.0 divisions as the titration proceeds. As shown in Figure 2, the experimentally observed limit is at least 6.4 divisions; this implies that the fluorescence intensity of terbium bound at the "weak" site is at least 83% of that at the "strong" site, and it almost certainly eliminates the possibility that the two sites differ by a factor of 3. In the calculations below we shall assume that the two sites have identical fluorescence properties, because even a 20% difference will have only a slight effect on the outcome.

The observation of the effect of the bound iron on terbium–transferrin fluorescence has been confirmed in a series of separate experiments involving monoferric transferrin, monogallic transferrin, and apotransferrin. The relative fluorescence intensities are not significantly affected by changing

the order of addition of the metal ions or by moderate changes in the degree of saturation of transferrin with metal (range of iron saturation 45–75%; gallium likewise; range of terbium saturation 10–35%), or by variations in the concentration of the sample solutions. In particular, the fluorescence of monogallium monoterbium transferrin appears to be identical with that of monoterbium transferrin. Practically identical results are obtained in 5 mM $NaHCO_3$, 30 mM Hepes, pH 8.2, and in 25 mM $NaHCO_3$, pH 8.3. Gallium and terbium bind to transferrin with comparable dissociation constants in 25 mM $NaHCO_3$; the iron complex is much more stable.

It was observed that the terbium-binding avidity of some apotransferrin solutions declined after several days' storage at 5 °C. This may be due to the leaching of metal ions such as zinc, calcium, or aluminum from the walls of the container. Only fresh solutions were used in this study.

Discussion

As shown in Figure 1, the fluorescence intensity of terbium transferrin is 2.8 times that of monoferric monoterbium transferrin. Control experiments, including that described in Figure 2, indicate that this number represents the ratio of unquenched to quenched fluorescence intensity, F_0/F, for a terbium ion bound to one metal-binding site on transferrin and a ferric ion at the other.

This ratio, $F_0/F = 2.8$, provides a measure of the separation between the metal-binding sites, R, in terms of the critical distance R_0 (Förster, 1948, 1965)

$$R = \left(\frac{F_0}{F} - 1\right)^{-1/6} R_0 \qquad (1)$$

yielding $R = 0.91R_0$.

The critical distance may be estimated from other data, since

$$R_0^6 = 8.78 \times 10^{-25} \kappa^2 Q n^{-4} J \text{ cm}^6 \qquad (2)$$

where κ^2 is the dipole–dipole orientation factor, Q is the donor quantum yield in the absence of energy transfer, n is the refractive index of the intervening medium, and J is the spectral overlap integral.

$$J = \frac{\int_0^\infty F(\nu)\epsilon(\nu)\nu^{-4}\mathrm{d}\nu}{\int_0^\infty F(\nu)\mathrm{d}\nu} \qquad (3)$$

where $\epsilon(\nu)$ is the molar absorptivity of the acceptor. It has been proposed (Latt et al., 1970) and recently demonstrated in one case (Berner et al., 1975; Horrocks et al., 1975) that many metal ion donor–acceptor pairs will behave as isotropic emitters and absorbers, so that the value of the orientation factor κ^2 may be taken to be $\frac{2}{3}$. Our measurements of the spectral overlap integral yield $J = 9.65 \times 10^{-15}$ M^{-1} cm^3. Using Luk's estimate of the quantum yield, $Q = 0.24$, and $n = 1.36$ (Luk, 1971; Horrocks et al., 1975), we find $R_0 = 27.1$ Å, in agreement with Luk (1971).

The uncertainty in R_0 is predominately due to the uncertainty in the quantum yield, Q, which Luk (1971) estimated as $Q = 0.24 \pm 0.06$. This leads to the estimate that the uncertainty in R_0 is 1.2 Å. If this uncertainty is taken to be equal to the standard deviation, then it is reasonable to expect that the true value of R_0 lies within two standard deviations of the calculated value, so that $R_0 = 27.1 \pm 2.4$ Å.

Equation 1 then yields for the distance between the sites $R = 25 \pm 2$ Å; it should be noted that this is not an upper or lower limit, but an approximation of the actual separation.

An estimate of whether the metal-binding sites lie at opposite ends of the transferrin molecule or relatively close together can be made from the following considerations; the protein can be roughly approximated as a prolate ellipsoid with 3:1 axial ratio (Bezkorovainy and Rafelson, 1964), having molecular weight 8×10^4 (Mann et al., 1970) and partial specific volume 0.725 cm^3/g (Oncley et al., 1947). Such an ellipsoid would be 40 Å thick and 120 Å long. Therefore, the metal-binding sites appear to be separated by a distance which is small in comparison to the linear dimensions of the macromolecule.

Acknowledgment

We thank Dr. D. C. Harris and Mr. T. B. Rogers for their advice and assistance.

References

Aasa, R., Malmstrom, B. G., Saltman, P., and Vanngard, T. (1963), *Biochim. Biophys. Acta 75,* 203.

Bates, G. W., and Schlabach, M. R. (1973), *J. Biol. Chem. 248,* 3228.

Berner, V. G., Darnall, D. W., and Birnbaum, E. R. (1975), *Biochem. Biophys. Res. Commun. 66,* 763.

Bezkorovainy, A., and Rafelson, M. E. (1964), *Arch. Biochem. Biophys. 107,* 302.

Dexter, D. L. (1953), *J. Chem. Phys. 21,* 836.

Fletcher, J., and Huehns, E. R. (1967), *Nature (London) 215,* 584.

Förster, Th. (1948), *Ann. Phys. (Leipzig) 2,* 55.

Förster, Th. (1965), in Modern Quantum Chemistry, Part III, Sinanoglu, O., Ed., New York, N.Y., Academic Press, p 93.

Harris, D. C. (1977), *Biochemistry 16,* 560.

Harris, D. C., and Aisen, P. (1975), *Biochemistry 14,* 262.

Harris, D. C., Gray, G. A., and Aisen, P. (1974), *J. Biol. Chem. 249,* 5261.

Holmberg, C. G., and Laurell, C. B. (1945), *Acta Physiol. Scand. 10,* 307.

Horrocks, W. D., Holmquist, J. B., and Vallee, B. L. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4764.

Latt, S. A., Auld, D. S., and Vallee, B. L. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1383.

Luk, C. K. (1971), *Biochemistry 10,* 2838.

Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970), *Biochemistry 9,* 1348.

Oncley, J. L., Scatchard, G., and Brown, A. (1947), *J. Phys. Colloid Chem. 51,* 184.

Thiers, R. E. (1957), in Methods of Biochemical Analysis, Vol. V, Glick, D., Ed., New York, N.Y., Interscience, p 273.

Windle, J. J., Wiersema, A. K., Clark, J. R., and Feeney, R. E. (1963), *Biochemistry 2,* 1341.