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Resolution of the Two Metal Binding Sites of Human Serum Transferrin by Low-Temperature Excitation of Bound Europium(III)†

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ABSTRACT: Derivatives of apotransferrin have been prepared in which europium replaces iron at either one or both of the two metal ion binding sites. At low temperature (77 K), pH 7.0, two sharp absorption lines are seen by means of laser-induced fluorescence of the bound europium. The one at 579.88 nm (17 245 cm^{-1}) is assigned to the C-terminal region A site, and the other at 579.26 nm (17 263 cm^{-1}) is assigned to the N-terminal region B site. The lifetimes of the excited $^5D_0$ states are 210 ± 20 and 310 ± 30 μs for the A and B sites, respectively. The energy difference between the two peaks is a function of pH, with the splitting decreasing from 0.62 nm (18.5 cm^{-1}) at pH 7.0 to 0.15 nm (4.5 cm^{-1}) at pH 8.0. This spectroscopic inequivalence may be explained by a charge difference of the liganding groups at sites A and B.

Human serum transferrin, M_r 80 000, is an iron transport protein composed of a single polypeptide chain and two metal ion binding sites (Aisen & Listowsky, 1980). Preliminary X-ray studies show the protein to be composed of two homologous domains, each containing a small cleft in which binding may occur (DeLucas et al., 1978). Iron may be substituted by a variety of divalent and trivalent metal ions including the lanthanide Eu(III) (Luk, 1971). Designated A (C-terminal region) and B (N-terminal region), the two sites are very similar but not identical in their metal binding properties. Some of the subtle differences observed by a variety of spectroscopic and chemical techniques are (1) a chelate specificity in which $Fe(NTA)_2$[1] directs iron to the A site at low pH (Harris, 1977) and $Fe(citrate)_4$ directs iron to the B site at neutral pH (Zapolski & Princiotto, 1977), (2) a factor of 5 difference in the iron binding constants at physiological pH ($K_{site\,A} = 5K_{site\,B}$) that increases to a factor of 30 at lower pH (Aisen et al., 1978), (3) spectroscopic inequivalence as detected by VO(II) and Cu(II) EPR (Cannon & Chasteen, 1975; Zweier & Aisen, 1977), and (4) observation of in vivo predominance of iron at site B (Leibman & Aisen, 1979). It was our intent to characterize this difference by a new technique for probing metal binding sites in proteins, Eu(III) excitation spectroscopy.

The Eu(III) excitation technique was introduced by Horrocks & Sudnick (1979a,b) in their study of the Ca^{2+} binding protein thermolysin. Under the conditions of their experiments, Eu(III) replaced Ca(II) in three of the four binding sites, and excitation spectra assignable to two distinct environments were observed. The usefulness of the europium ion spectroscopy arises from a unique electronic transition, $^7F_0 \rightarrow {}^5D_0$. Because both the ground and excited states are inherently nondegenerate, the field due to the ligands can never split the transition. In a homogeneous environment an absorption curve of Lorentzian shape should be observed for the $^7F_0 \rightarrow {}^5D_0$ transition. In fact, the absorption coefficient of this highly forbidden transition is so small ($\epsilon \sim 0.001$ M^{-1} cm^{-1}) that the absorption has never been observed directly but only by fluorescence excitation. Although the degeneracy of the transition is independent of ligand field effects, the energy is not. Sites with different ligand fields will differ slightly in the positions of their resonances. Any structure in the profile is therefore due to heterogeneity of the sites. Thus, by measuring the fluorescence excitation spectrum of transferrin in which Eu(III) has been substituted for Fe(III), we were able to identify and resolve transitions due to the two different sites.

Experimental Procedures

Sample Preparation. Apoprotein samples from Calbiochem Behring were used without further purification after gel electrophoresis showed them to contain no contaminants. Concentrations used were 0.2–1.0 mM. Europium(III) chloride hexahydrate (Aldrich, 99.99% pure) was dissolved in water to 25–32 mM. Small aliquots were added to the protein to achieve the desired saturation.

The buffer used for room temperature spectra contained 10 mM Hepes and 5 mM $NaHCO_3$ at pH 7.0. Low-temperature spectra used a buffer of 100 mM Hepps, 10 mM $NaHCO_3$, and 5% sucrose at pH 7.0–8.0. Hepps was chosen for the low-temperature experiments for two reasons: (1) with a pK_a = 8.00, it has a $\Delta pK_a/\Delta T$ value of −0.007 and is therefore resistant to pH changes upon cooling; (2) Hepps does not bind metals and thus will not interfere with metal complexation to the protein and will not introduce adventitious metals into the solutions. The presence of sucrose in the buffer made the protein more resistant to denaturation upon freezing and thawing.

Dieuropic samples were prepared by adding 1.9[M] equiv (where [M] is the protein concentration) of europium stock to the apoprotein sample. Europium was specifically loaded at the A site of transferrin by first incubating the protein with

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[1] Abbreviations: NTA, nitrilotriacetate; $Eu_{A,B}Tf$, dieuropic transferrin; Eu_AFe_BTf, monoeuropic (site A) monoferric (site B) transferrin; Eu_BFe_ATf, monoeuropic (site B) monoferric (site A) transferrin; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; PMT, photomultiplier tube.

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Table I: Transition Energies and Line Widths for Europium(III) in Transferrin

species	T (K)	pH	resonance positions			line widths
			A_s	A	B	
$Eu_{A,B}Tf$	298	7.0		578.90 (17 274)[a]		0.95 (28)
	77	7.0	580.07 (17 239)			
				579.83 (17 246)		0.69 (20)
					579.57 (17 254)	
	77	8.0	*b*			
				580.24 (17 234)		0.37 (11)
					580.10 (17 238)	
Eu_AFe_BTf	77	7.0	580.13 (17 237)			
				579.88 (17 245)		0.60 (18)
	77	8.0	*b*			
				580.36 (17 230)		
Eu_BFe_ATf	77	7.0			579.26 (17 263)	0.45 (13)
	77	8.0			580.21 (17 235)	

[a] Transition energies and line widths are reported both in nm and in (cm^{-1}). [b] A clear assignment could not be made for these spectra.

1 equiv of $Fe(citrate)_4$, which loads the transferrin B site, dialyzing, and then adding 1 equiv of europium. The predominant species produced is Eu_AFe_BTf. Site B is specifically loaded with europium by incubation of the protein with $Fe(NTA)_2$. This loads the A site with iron so that added europium now goes to the B site. Here the predominant species is Eu_BFe_ATf.

In each experiment, europium blanks containing every component except protein were run. Both citrate and nitrilotriacetate were found to bind to europium and shift its resonance. We therefore added the lanthanide after dialysis, since adding the metal in the presence of chelators led to reduced EuTf formation. Europium in water at pH 7.0 was used as a calibration standard. At room temperature aquoeuropium(III) complex gives an absorbance at 578.85 nm that is 0.17 nm wide.

Low-temperature samples (0.50 mL) were contained in 5-mm Wilmad 528PP NMR tubes that did not crack at low temperatures. An optical liquid nitrogen containing Dewar flask was used for low-temperature studies.

Experimental Apparatus. A Quantel TDL(III) Nd:YAG pumped dye laser, tunable from 600 to 576 nm, with a 0.002-nm line width (Rhodamine 590–methanol as the solvent) was used to excite the Eu(III)–protein solutions. Emitted light of wavelengths greater than 600 nm was collected through an OG590 Schott glass filter by a red-sensitive PMT at 90° to the excitation pulse. A programmable Uniblitz shutter was also used to help protect the PMT from the large initial laser scatter that was unavoidable especially at low temperatures. Crossed zener diodes, clipped and diverted to ground, protected the rest of the circuit from laser transients (Horrocks & Sudnick, 1979b). By collecting the total light intensity with a boxcar averager (gated in time beyond the interfering laser scatter) and plotting the intensity as a function of excitation wavelength, we obtained excitation spectra. Temporal effects of the delay of the gate were also examined by measuring the excitation spectra at several different delay times. Finally, the lifetimes of the various peaks were measured with a multichannel scaler. The apparatus and timing sequences are discussed at length elsewhere (O'Hara, 1981).

Results

$Eu_{A,B}Tf$: Observation of Heterogeneity. The excitation spectra of $Eu_{A,B}Tf$ are shown in Figure 1. Room temperature spectra were somewhat asymmetric, suggesting, but not proving, heterogeneity. After the sample was cooled to 77 K, the envelope was resolved into three peaks. The entire spectrum also undergoes both a red shift and a general line narrowing. Both the shape and position of low-temperature spectra were found to be pH dependent as can be seen by comparison of the spectra at pH 7.0 and at pH 8.0. It is also interesting to note that the molar emission intensity increased by a factor of 5 on cooling to 77 K (Figure 1).

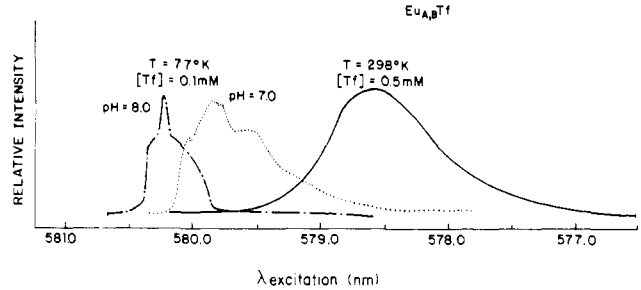

FIGURE 1: Excitation spectra of dieuropic transferrin ($Eu_{A,B}Tf$) at room temperature (solid line) and low temperature (dashed and dotted lines). Note the factor of 5 increase in emission ($[Tf]_{298} = 5[Tf]_{77}$) upon cooling.

Eu_AFe_BTf and Eu_BFe_ATf: Resolution of the Two Sites. The appearance of a structured absorption profile at low temperature meant that there must be some site-related heterogeneities. To test whether the heterogeneity arose from differences betwen sites A and B, we prepared specifically labeled europium transferrins by making use of the specificity of iron compounds for site A or B. Once iron was specifically loaded, the Eu(III) could then be added to the remaining site. Both Eu_AFe_BTf and Eu_BFe_ATf were prepared by using this strategy. The spectra of the labeled samples as well as the $Eu_{A,B}Tf$ sample are depicted in Figure 2. The B site environment induces a symmetric Eu(III) absorption profile 0.62 nm to the blue of the A site absorption. The A site absorption profile is not symmetric due to the presence of a low-energy shoulder, A_s, that was present in each spectrum containing A site Eu(III). The separation of A_s from the central peak also seemed to be pH dependent. The shoulder may be due to some heterogeneity within the A site that is pH dependent. The sum of the two individual spectra approximates quite well the dieuropic species spectrum. Data obtained at pH 8.0 are qualitatively similar, with a lower energy A site resonance that is somewhat asymmetric and a higher energy B site resonance. Quantitative differences include the actual energies as well as the energy differences; here the energy separation is reduced to 0.15 nm (4.5 cm^{-1}).

Table I reports line widths and energies of Eu(III) transitions that we have observed in transferrin under a variety of conditions. Table II lists selected Eu(III) transition energies

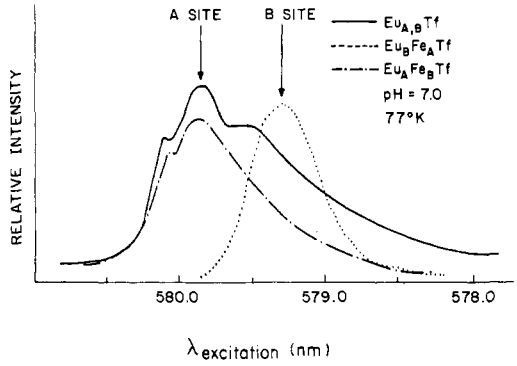

FIGURE 2: Resolution of the two metal binding sites as shown by the excitation spectra of various substituted transferrins at 77 K, pH 7.0.

Table II: $^7F_0 \rightarrow {}^5D_0$ Transition Energies

	pH	energy at 298 K (cm^{-1})	energy at 77 K (cm^{-1})
$Eu(H_2O)_{9-10}$	7.0	17 276	
Eu_3(parvalbumin)[a]	6.5	17 280	
		17 274	
		17 265	
		17 253	
Eu_3(thermolysin)[b]		17 268	
		17 247	
Eu_4(calmodulin)[c]	7.0	17 261	17 249
Eu_2(transferrin)	7.0	17 274	17 254
			17 246
			17 239
$Eu(citrate)_4$	7.0		17 256
$Eu(nitrilotriacetate)_2$	7.0		17 241
Eu(dipicolinic acid)[d]		17 266	
$Eu(dipicolinic\ acid)_2$[d]		17 247	
$Eu(dipicolinic\ acid)_3$[d]		17 232	
Eu in $LaCl_3$ (s)[e]			17 267
Eu in $Eu(C_2H_5SO_4)_3 \cdot 9H_2O$ (s)[f]			17 265
Eu in KLu_3F_{10}[g]			17 257
Eu in Y_2O_3 (s)[h]			17 216

[a] Rhee et al. (1981). [b] Horrocks & Sudnick (1979a). [c] This laboratory, unpublished work. [d] Sudnick (1980). [e] De Shazer & Dieke (1963). [f] Hellwege (1957). [g] Valon & Vedrine (1977). [h] Chang & Gruber (1964).

that have been measured in other relevant systems and summarizes the transferrin data.

Lifetimes of A and B Sites. Previous work on thermolysin demonstrated the acute dependence of the spectra on several experimental parameters, most specifically the boxcar delay time, i.e., the initial period during which no data are collected (Sudnick, 1980). Although necessary to gate out the scattered laser light, our use of a 0.54-ms delay time could possibly produce time discrimination if the sites have different lifetimes. We therefore measured the excitation spectra of $Eu_{A,B}Tf$ at seven different delay times (Figure 3). The relative intensities of A and B sites changed with delay time, suggesting different time dependencies, i.e., different lifetimes for Eu(III) in the two sites.

The lifetime for Eu(III) in each site was measured by positioning the laser to excite specifically the A (580.35 nm) or B (580.05 nm) site and monitoring the emission as a function of time. Figure 4 compares the radiative decay rates or slopes obtained by selective excitation. The lifetimes measured for the two sites were as follows: "A" site, 210 ± 20 μs; "B" site, 310 ± 30 μs. These are not pure A or B site lifetimes since our conditions did not preclude some excitation of the other site. The difference in lifetimes may be due to some difference in solvent H_2O coordination since this is the major source of nonradiative decay (Haas & Stein, 1971).

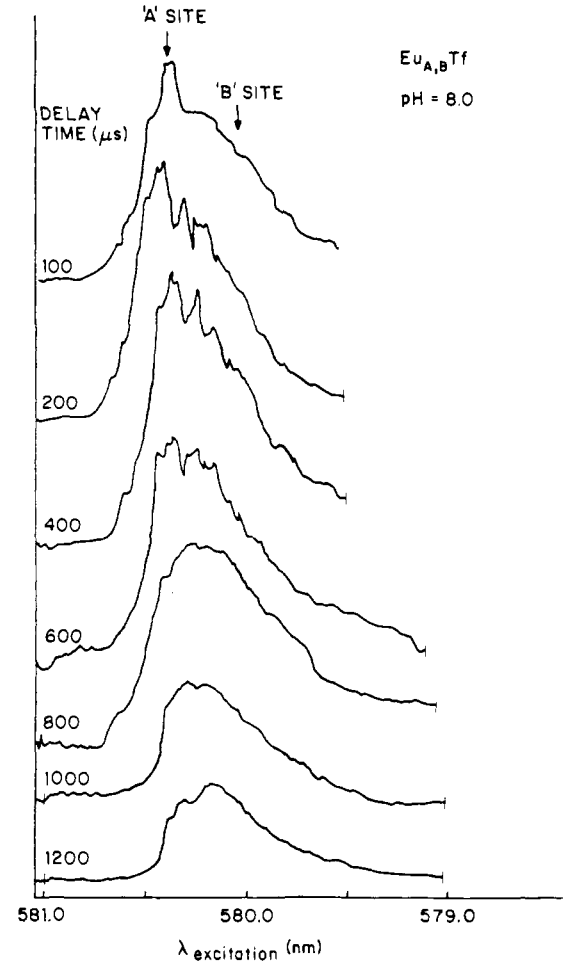

FIGURE 3: Effects of the delay time on emission spectra for dieuropic transferrin, pH 8.0, at 77 K. A and B site resonances were assigned from the specifically labeled mixed metal transferrins.

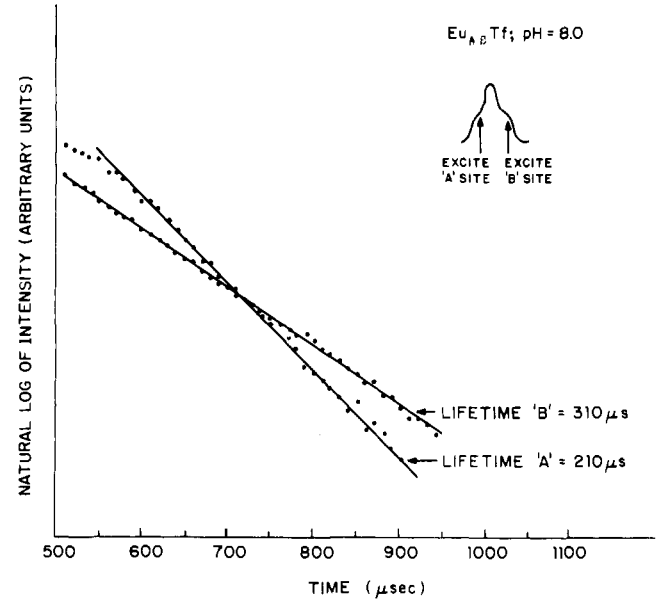

FIGURE 4: Lifetime measurements for excitation of the "A" and "B" sites, pH 8.0, 77 K, of dieuropic transferrin ($Eu_{A,B}Tf$).

Discussion

Intensity Variation with Temperature. A factor of 5 increase in intensity was observed in going from room temperature to 77 K. Part of this change is due to the usual decrease

of nonradiative quenching rates with decreasing temperature. A significant part is also due to the fact that the $^7F_1$ state that is $\sim$200 cm^{-1} above the ground $^7F_0$ state is well populated at 295 K but almost empty at 77 K.

Line Widths. Figure 2 illustrates the spectroscopic inequivalence of Eu(III) in the two sites of transferrin. At pH 7.0 and 77 K Eu(III) transition energies at sites A and B differ by 18.5 cm^{-1}. This difference is unfortunately only a little more than the width of each transition. Thus, while the spectra of Eu_BFe_ATf and Eu_AFe_BTf are clearly different, when they are, in effect superimposed, as in $Eu_{A,B}Tf$, the spectrum is only partially resolved into contributions from the two different sites. Exactly the same problem of resolution of spectra at inequivalent sites was found for Eu(III) bound to parvalbumin (Rhee et al., 1981) and thermolysin (Snyder et al., 1981).

There may be both homogeneous and heterogeneous contributions to the line width at the individual A or B sites. The homogeneous broadening caused by photon interactions necessarily goes to zero as $T \rightarrow 0$ with the very small radiative decay rate being the only remaining cause of homogeneous broadening. It has been shown experimentally for Eu(III) in glasses (Selzer et al., 1976; Avouris et al., 1977) that the homogeneous width that is on the order of several cm^{-1} at 298 K decreases as the temperature is lowered but the drop is particularly steep between 77 and 4 K. The heterogeneous broadening reflects the variety of local fields caused by the numerous local conformations of the protein molecule. An important question in protein chemistry is whether the protein is able to relax into the presumably small number of conformations available at equilibrium at 4 K or whether a glassy disorder is already frozen in at a higher temperature. Present data do not permit firm conclusions as to the origin of the line widths.

$^7F_0 \rightarrow {}^5D_0$ Transition Energies. What is the meaning of the difference of the $^7F_0 \rightarrow {}^5D_0$ absorption energies of Eu(III) at the A and B sites of Tf at 77 K? To answer this question it is helpful to look at the range of transition energies shown in Table II. There is a general qualitative trend pointed out by Sudnick (1980) for the transitions to be shifted to lower energies as the positive charge on the Eu(III) complex decreases. For example, $[Eu(III)(H_2O)_9]^{3+}$ has close to the highest observed transition energy. Ionic crystals in which the Coulomb energy stabilizes a high positive charge have high transition energies. On the other hand Eu(III) surrounded by oxide ions (in Y_2O_3) has the lowest observed transition energy. Adding dipicolinate dianions diminishes successively the transition energy.

On the basis of the qualitative trend just discussed, we can say that the Eu(III) at site A has a more negative environment than at site B. The exact nature of this electrostatic charge difference is not known. When the pH is increased from 7 to 8, there are substantial red shifts at both sites with the B shift being greater. One possible interpretation is that in this pH range a proton is lost either by the obligatory HCO_3^- or by a nearby tyrosine or imidazolium ion which subsequently ligates the metal. The greater breadth of the A site absorption at pH 7 may be due to the fact that both the protonated and deprotonated forms are present at this pH in contrast to that to pH 8.

An Application of the Site Resolution. In this work we have shown that the technique of Horrocks and co-workers of fluorescence excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition in Eu(III) can be applied to the resolution of inequivalent sites on an iron carrier protein in which Eu(III) is substituted for Fe(III). It is possible that further development of this technique might lead to a reliable analytical method for the relative occupancy of A and B sites by Fe(III) in human plasma transferrin.

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