Optical Activity of Terbium Ions Bound to Transferrin and Conalbumin Studied by Circular Polarization of Luminescence†

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ABSTRACT: The optical activity of terbium ions bound to transferrin and to conalbumin has been studied by the circular polarization of the luminescence of the bound ions. The anisotropy factor is very large (reaching a value of 0.23 at 540 nm). Due to the multitude of electronic transitions responsible for the emission spectrum, the anisotropy factor varies markedly with wavelength and shows fine details. The optical activity has thus been used as a sensitive indicator for probing the asymmetry of the binding sites for the metal ions. The emission spectrum and the spectrum of the anisotropy factor are extremely similar for terbium ions bound to transferrin and to conalbumin, reflecting similarity in structure and conformation of the binding sites of the two proteins. The same spectrum was observed for the anisotropy factor of transferrin–Tb^{3+} complexes of different degrees of saturation, and for ternary complexes of the type transferrin–Ho^{3+}–Tb^{3+} and transferrin–Fe^{3+}–Tb^{3+} of various stoichiometric ratios. These results strongly suggest that under the conditions studied (aqueous solution, pH 8.5, room temperature) the two metal binding sites of a transferrin molecule are equivalent in structure and conformation.

The storage and transport of iron in living systems are performed by an elaborate system, in which proteins of high affinity for iron play an important role (Aisen, 1970). Transferrin from blood serum and conalbumin from egg white are two examples. These are glycoproteins of about 80,000 molecular weight and apparently comprised of a single polypeptide chain. There are two binding sites per protein molecule which have identical or similar affinity for ferric ions. Two histidine and three tyrosine residues were shown to participate in the metal binding sites of transferrin (Bezkorovainy and Grohlich, 1971). The iron ions can be replaced by a variety of other transition metal and rare earth ions. The replaced ions have proved to be very useful as paramagnetic or fluorescent probes for the study of the metal binding sites of transferrin and conalbumin (Aisen et al., 1969; Price and Gibson, 1972; Luk, 1971).

The binding of trivalent terbium ions to transferrin has been recently studied by Luk (1971) and Teuwissen et al. (1972). Two ions bind per protein molecule, the terbium being highly luminescent in the complex. The emission spectrum is composed of four quite narrow bands (with some fine structure) caused by internal transitions in the 4f shell of the metal. These transitions are essentially forbidden; the corresponding absorption bands are therefore extremely weak and the luminescence lifetime long (about 1.3 msec) (Luk, 1971). The excitation of the terbium ions is affected by energy transfer from the tyrosine residues of the protein which are situated near the bound ions (Luk, 1971).

The optical activity of metal absorption bands in metalloproteins has been extensively used to investigate the binding site of the metal (Vallee and Wacker, 1970). Due to the very weak intensity of the absorption bands of the terbium ions in the visible spectral range, the study of the circular dichroism (CD) of the absorption bands of this ion when bound to proteins is impracticable. It has been shown recently, however,

that the circular polarization of luminescence (CPL) is also a manifestation of asymmetry of chromophores (Gafni and Steinberg, 1972; Steinberg and Gafni, 1972; Emeis and Oosterhoff, 1971; Gafni et al., 1973; Steinberg, 1974). More specifically, the CPL of a chromophore is related to its asymmetry in the emitting excited state in the same way that its CD is related to the asymmetry in the ground state. For the study of CPL the forbidden nature of the relevant transitions may even turn out to be an advantage since weak transitions may assume very high optical activity (Kuhn, 1958).

In the following we present the behavior of the circular polarization of the luminescence of trivalent terbium ions bound to transferrin and to conalbumin. Since the luminescence involves many electronic transitions, the CPL spectrum exhibits an intricate pattern, which renders the CPL a sensitive fingerprint of the environment of the ion. A comparison of the metal binding sites of the two proteins and of the two sites in a single protein is thus facilitated.

Experimental Section

Human serum transferrin and avian egg white conalbumin, both iron free, were purchased from Sigma Chemical Co. $TbCl_3$ and $HoCl_3$ were obtained from Alfa Inorganics and were of 99.99% purity. $FeCl_3$, Analytic Grade, was a product of Fischer Scientific Co. These materials were used without further purification.

Solutions of tranferrin and conalbumin were prepared in 0.05 M Tris buffer (pH 8.5) which contained 5×10^{-3} M sodium bicarbonate. Protein concentrations were about 10^{-4} M.

Complexes of the inorganic salts with the proteins were usually prepared by adding the appropriate volume of metal salt solution in double distilled water (usually 0.025 ml) to the protein solution (usually 0.5 ml) with mild stirring. The concentration of the salt solution was adjusted to yield the desired ratio of metal–protein in the final complex.

Triple complexes of the type transferrin–Tb^{3+}–Ho^{3+} were prepared by adding a solution of $HoCl_3$ to a solution of the
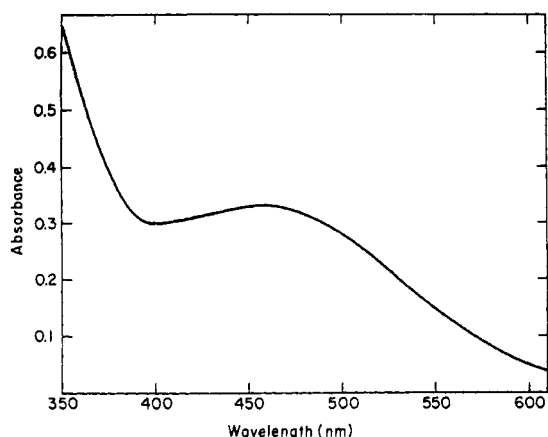
FIGURE 1: Absorption spectrum of transferrin-iron prepared by adding 16 μl of a solution of $FeCl_3$ in water (10^{-2} M) to 1 ml of a solution of transferrin (8 × 10^{-5} M) in Tris buffer containing 5 × 10^{-3} M sodium bicarbonate (pH 8.5); light path 1.0 cm; room temperature (~23°).

transferrin-Tb³⁺ complex (1:1). The amount of Ho³⁺ added was fivefold that of Tb³⁺ present.

Triple complexes of the type transferrin-Tb³⁺-Fe³⁺ or conalbumin-Tb³⁺-Fe³⁺ of different stoichiometric ratios were prepared in three different ways: (a) a mixture of the two cations in the desired ratio was added to a solution of the apoprotein, the total number of cations of heavy metal being equal to the number of protein binding sites; (b) Fe³⁺ was added to a transferrin-Tb³⁺ complex of ratio 1:2 to replace part of the Tb³⁺; (c) Fe³⁺ and transferrin were mixed in a molar ratio of 1:1 and allowed to react for 12 hr. Tb³⁺ was then added to populate a part of the free binding sites.

In view of the complications observed by Bates and Schlabach (1973) in the binding of ferric ions to transferrin under certain conditions, the spectra of transferrin-Fe³⁺ complexes of different stoichiometric ratios, prepared as described above, were studied at various intervals after mixing the constituents. Figure 1 shows the spectrum of a transferrin-Fe³⁺ mixture of molar ratio 1:2, 20 hr after preparation. Similar spectra, but of proportionately lower intensity, were obtained for lower degrees of saturation. The absorption developed most of its intensity within 2 hr after mixing the constituents and was practically constant after 20 hr. It may be noted that the spectrum of the transferrin-Fe³⁺ complex is similar in shape to that reported by Bates and Schlabach for fully saturated transferrin though it is somewhat lower in intensity. Thus, most of the iron (at least 80%) binds at the specific sites of the protein under the conditions used in this study. As regards the transferrin-Tb³⁺ complexes, we have observed by fluorimetric titrations that two terbium ions bind per protein molecule, in agreement with the studies reported by Luk (1971).

The solutions were studied on the day of preparation, at least 1 hr after preparation of complexes containing protein and lanthanide ions and 3 hr after preparation of complexes containing iron. Very similar results were, however, obtained 1 week after preparation, the solutions being stored in the meantime at 4°.

All measurements were carried out at room temperature (~23°).

Measurements of circular polarization of luminescence were performed with an instrument built in our laboratory. The molecules under study are excited by unpolarized mono-chromated light, and the circularly polarized component of
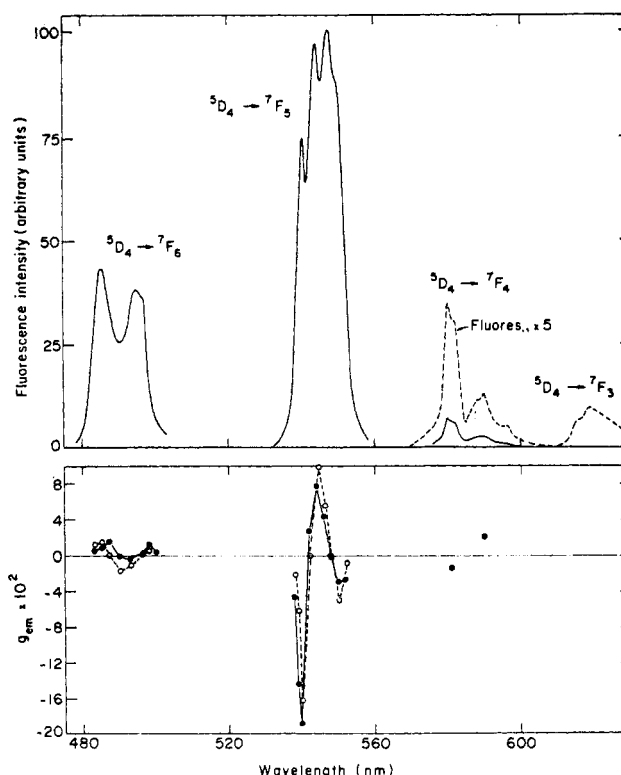

FIGURE 2: Fluorescence spectra and the spectra of the emission anisotropy factor, g_{em}, of the Tb³⁺ complexes of transferrin and conalbumin. The ratio of metal:protein is 2; solvent, 0.05 M Tris buffer (pH 8.5) and 5 × 10^{-3} M sodium bicarbonate; temperature, ~23°; spectral bandwidth of the emission monochromator, 1.6 nm. Upper part: emission spectra (identical for the two metal-protein complexes). Lower part: spectrum of the emission anisotropy factor: (—) transferrin-Tb³⁺; (---) conalbumin-Tb³⁺.

the emitted light is selectively modulated by an elasto-optic light modulator and detected by a photomultiplier. The ac and dc components of the electric signal generated by the modulated beam after monochromatization are amplified and monitored. Full details of the instrument are described elsewhere (Steinberg and Gafni, 1972). The luminescence was excited by the emission bands at 290–305 nm of a 100-W high-pressure mercury arc (Osram, HBO 100 W/2). The spectral bandwidth of the excitation monochromator was about 30 nm, while the spectral resolution of the emitted light varied from 0.5 to 1.6 nm. The CPL is expressed by the anisotropy factor for emission defined as $g_{em} = \Delta F/(F/2)$, where F is the total luminescence intensity and ΔF is the intensity of the circular polarized component of the luminescence. ΔF is assigned a plus sign for left-handed circular polarization.

Fluorescence spectra were obtained from the dc signal of the above instrument for measuring CPL. These spectra were not corrected for the response of the instrument. This, however, will not distort the shape of the emission bands to any significant extent because of the narrow spectral range of the bands.

Results and Discussion

Optical Activity of Tb³⁺ Bound to Transferrin and Conalbumin. The luminescence and CPL spectra of the terbium ions in the complexes transferrin-Tb³⁺ and conalbumin-Tb³⁺ are presented in Figure 2. The metal binding sites were saturated with Tb³⁺ cations. The emissions spectra presented in Figure 2 are composed of several narrow bands each of which

TABLE I: Emission Anisotropy Factor, g_{em}, of Transferrin–Tb^{3+} of Molar Ratio $1:2$.[a]

	Emission Wavelength (nm)							
	487	540	540.5	541	544	548	550	580
$g_{em} \times 10^2$	+1.47	−23.0	−20.0	−9.1	+8.4	0.0	−3.6	−3.7

[a] Spectral bandwidth of the emission monochromator, 0.5 nm. Except for the spectral bandwidth, the conditions are the same as those described in Figure 2.

shows fine structure. The bands stem from electronic transitions from the $^5D_4$ atomic level to several ^7F levels of different J values (Ofelt, 1963; Thomas et al., 1963). These levels are split by the ligand field into components whose number and separation depend on the nature and symmetry of the environment of the cation (Filipescu et al., 1964). The various emission lines therefore result from independent electronic transitions. Since each of the electronic transitions is associated with its own electric and magnetic transition dipole moments, the optical activity may markedly change from transition to transition, which is the reason for the pronounced variation of the anisotropy factor with wavelength observed for the protein–terbium complexes. The exceptionally large values obtained for the emission anisotropy factor at a few wavelengths are noteworthy. (Some of them, measured at a high spectral resolution of 0.5 nm, are presented in Table I.) This is made possible by the forbidden nature of the transitions. The wealth of detail in the CPL spectra of the protein–Tb^{3+} complexes, the large signals obtained, and the high sensitivity of the optical activity to the nature of the environment of the chromophores involved render CPL a very sensitive tool for probing the metal binding sites.

The emission spectra of the terbium ions in transferrin–Tb^{3+} and conalbumin–Tb^{3+} are identical. Still more striking is the close similarity in the CPL spectra of the two protein–Tb^{3+} complexes, which, except for a slight relative shift in wavelength, are of very similar shape and of comparable magnitude. It is very unlikely that the similarity between the CPL spectra of the two protein–metal complexes is a coincidence, since each of the spectra is made up of a large number of electronic transitions, each with distinct electric and magnetic transition dipole moments which are very sensitive in the present case to pertubations by the environment because of the forbidden nature of the transitions. The binding sites of the two proteins thus seem to offer nearly identical asymmetric environments to the bound metal ions. Despite the close similarity between the CPL spectra of the two proteins, small differences do occur and possibly reflect differences in sequence and folding of the two proteins in regions removed from the immediate surroundings of the bound metal ions.

Optical Activity of Bound Tb^{3+} on Partial Saturation of the Protein Binding Sites with Metal Ions. There is a controversy as to the equivalence of the two metal binding sites of a molecule in the iron binding proteins. The following experiments were performed in relation to this problem.

The CPL of a few complexes of terbium with transferrin of different degrees of saturation was measured at a few wavelengths. The molar ratios of Tb^{3+}:protein were 0.6, 1.1, 2, and an excess of 5. The CPL was measured at 487, 540, and 544 nm. The values of the anisotropy factor obtained for the various Tb^{3+}:protein ratios at each wavelength were identical.

These results show that one or more of the following circumstances hold. (1) The sites are identical in their asymmetry.

(2) The sites have equal affinity for the Tb^{3+}; the metal thus populates the two sites equally at any level of saturation. (3) The binding of the terbium to the sites is highly cooperative; the Tb^{3+} ions thus populate the protein in an all-or-none fashion. Here again the observed optical activity will not depend on the extent of saturation even if the two sites of a protein molecule are of different asymmetry. As discussed above, equal optical activity for the two sites strongly suggests in our case that the sites are identical in constitution and structure. Alternative 1 above thus suggests that the two binding sites on a protein molecule are identical, while alternatives 2 and 3 may imply nonequivalent sites.

The optical activity of a ternary complex of the type transferrin–Tb^{3+}–Ho^{3+} was investigated. This complex was prepared by adding a fivefold excess of Ho^{3+} to a $1:1$ complex of transferrin and Tb^{3+}. Ho^{3+} has a higher affinity for transferrin than Tb^{3+} and is nonfluorescent under the present experimental circumstances (Luk, 1971). Indeed, on adding Ho^{3+} ions to the transferrin–Tb^{3+} complex the fluorescence dropped in intensity by nearly an order of magnitude, but the anisotropy factor (measured at 540 and 554 nm) remained essentially the same. These results render somewhat unlikely alternative 2 discussed above, namely that the two sites on a transferrin molecule are nonequivalent but happen to have equal affinity for Tb^{3+}. The experiments with the ternary complexes will require that the sites also have equal affinity for Ho^{3+}.

In order to test alternative 3 discussed above, namely that the sites are nonequivalent but that the binding of Tb^{3+} at the two sites of transferrin is cooperative, the optical activity of ternary complexes of transferrin with ferric and terbium ions of different molar ratios was studied at a few wavelengths. The complexes of transferrin–Tb^{3+} with the following molar ratios were tested: $1:1:1$, $1:0.33:1.67$, $1:0.5:1$, and $1:0.8:1$.[1] The wavelengths selected were 540 and 544 nm for the first two complexes and 487, 540, 544, and 550 nm for the last two complexes. The complexes were prepared by a variety of methods as described in the Experimental Section. The same anisotropy factor and hence the same optical activity were obtained for the luminescence of the terbium throughout. (Similar experiments with conalbumin–metal complexes of the type conalbu-

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[1] Free Tb^{3+} in solution is not excited under the conditions used, while Tb^{3+} bound at the specific site of transferrin is rendered highly luminescent by transfer of excitation energy from an adjacent tyrosine residue. It is of interest to note that negligible fluorescence of the terbium ions was observed when added to transferrin which was saturated with iron. The terbium is thus obviously excluded by the iron from the specific binding site of the protein. The possibility cannot be excluded that nonspecific binding occurs at other sites which do not provide a proper chromophore for excitation of the Tb^{3+} by energy transfer. This possibility does not affect, however, the conclusions drawn below, since nonspecific binding, if it occurs at all, does not affect the measurements.

min–Tb^{3+}–Fe^{3+} of molar ratios 1 : 0.5 : 1 and 1 : 0.8 : 1 showed the same optical activity as saturated conalbumin-Tb^{3+}.) The binding of ferric ions to transferrin is known to be noncooperative (Lane, 1971). It is bound with much higher affinity than terbium (Aisen, 1970; Luk, 1971). Furthermore, once bound the reequilibration of the ferric ions among the sites is negligible even for periods of days (Aisen and Leibman, 1968). Thus, when ferric ions bind to the transferrin below saturation levels, they block randomly and irreversibly (for our purposes) the sites they occupy. A fraction of the protein molecules thus have only a single site available for binding of Tb^{3+} ions. For these sites cooperative binding of Tb^{3+} is of course impossible. Thus, the explanation that cooperativity of binding is the cause of the invariability of the optical activity of the terbium with degree of saturation of the protein may be discarded.

To summarize, the two metal binding sites of a transferrin molecule seem to be equivalent. The possibility that they are nonequivalent but of equal affinity for each of the metal ions tested cannot be discarded but seems unlikely. The binding of terbium ions to the metal binding sites is not cooperative.

Electron paramagnetic resonance (epr) studies of iron and chromium complexes of transferrin and conalbumin at very high protein concentrations (10–14%) and very low temperature (77 and 103°K) have indicated differences between transferrin and conalbumin binding sites and two types of metal complexes with each protein (Price and Gibson, 1972; Aisen *et al.*, 1969). Due to the markedly different conditions under which the epr studies and the present investigation were conducted, a comparison between the conclusions drawn in the previous and the present studies seems to be of little meaning.

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