ATTACHMENT OF FLUORESCENT METAL CHELATES TO MACROMOLECULES USING "BIFUNCTIONAL" CHELATING AGENTS

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SUMMARY: The use of "bifunctional" chelating agents to covalently attach stable chelates of terbium and europium to human serum albumin results in products whose lanthanide fluorescence may be studied easily at micromolar concentrations with standard instrumentation. The lanthanide ions may be added specifically and quantitatively to the protein-bound chelating groups in 0.1 M citrate, pH 6.5. The use of these reagents should greatly reduce ambiguities in the determination of distances between sites on macromolecules by energy transfer measurements.

INTRODUCTION: Fluorescence techniques have been used to study the structural and dynamic properties of macromolecules in several different ways (1). Particularly interesting is the determination of intramolecular distances in macromolecules by measuring the efficiency of energy transfer between a fluorescent "donor" and a suitable acceptor. In Förster's theory (2) of dipole-dipole energy transfer, the distance R_o (at which the singlet-singlet energy transfer efficiency is 50%), is given by

$$R_o = (JK^2Q_on^{-4})^{1/6}(9.97 \times 10^{-3})\ \text{Å} \qquad (1)$$

where K^2 is the orientation factor for dipole-dipole transfer, Q_o is the quantum yield of the donor in the absence of transfer and n is the refractive index of the medium. The spectral overlap integral J is given by

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\int F(\lambda)d\lambda} \qquad (2)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ and $\epsilon(\lambda)$ is the extinction coefficient of the energy acceptor at that wavelength.

Förster's theory further predicts that the rate constant for dipolar energy transfer is proportional to R^{-6}, where R is the distance between donor and acceptor. The efficiency E of transfer for a donor-acceptor pair

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at a defined distance and orientation is given by

$$E = R^{-6}/(R^{-6} + R_o^{-6}) \quad (3)$$

This theory has been tested in well-defined model systems (1,3). Recently, singlet-singlet energy transfer has been used not only in determining distances in various biological macromolecules (4,5), but also to study stoichiometry, conformation, and kinetics of complex formation in associating protein systems (6,7).

As pointed out by Dale and Eisinger (8-10), the results of energy-transfer experiments involving aromatic molecules (such as dansyl derivatives) may suffer from considerable ambiguity due to lack of knowledge about the dipolar orientation factor K^2. This results from the highly polarized nature of the absorption and emission of radiation by individual organic molecules. Vallee and co-workers (11,12) have pointed out the fact that, due to the near degeneracy of several energy levels, electronic transitions in many metal complexes are practically unpolarized. This property can reduce or remove uncertainties in the value of K^2. Experimental results obtained for energy transfer from a fluorescent terbium ion to a cobalt ion, when both metals are bound to specific sites on the enzyme thermolysin, support the expectation that energy transfer between such metal ions is not subject to orientation effects (13,14).

Recently, we have prepared a new class of compounds, termed "bifunctional" chelating agents, which can form stable chelates with metal ions and can also bind to sites on biological molecules (15,16,17). As illustrated in Fig 1, these compounds are close analogs of EDTA, with a para-substituted phenyl group attached to C-1. Because of their structural similarity to EDTA, these chelating agents are expected to form a large number of metal complexes which are stable under physiological conditions in aqueous solution.

In this report we describe the covalent attachment of chelating agents and chelates of terbium (III) and europium (III) to various amino acids

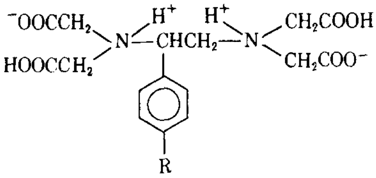

Figure 1. The structure of the "bifunctional" chelating agents employed in this study ($R = NO_2$, NH_2, or N_2^+).

and to the protein human serum albumin. The products are stable under physiological conditions at submicromolar concentrations, and they exhibit easily measurable lanthanide fluorescence. The use of such metal complexes in energy-transfer experiments should significantly reduce the uncertainty involved in estimating the separation between donor and acceptor.

MATERIALS AND METHODS: Except where noted, all reagents were purchased commercially and used without further purification. Chromatographic columns and materials were obtained from Bio-Rad Laboratories. Terbium chloride ($TbCl_3 \cdot 6H_2O$, 99.9%) and europium chloride ($EuCl_3 \cdot 6H_2O$, 99.9%) were purchased from Alfa Inorganics Inc. Human serum albumin was obtained from Hyland Laboratories as a 25% aqueous solution. Precautions were taken to avoid contamination by extraneous metal ions (17). Bifunctional chelating agents (Fig 1, $R = NO_2$, NH_2, N_2^+) were prepared as described previously (16).

Metal chelates may be formed before or after attachment of a bifunctional chelating agent to a target molecule. The compounds N-acetyl-L-tyrosinamide, Nα-acetyl-L-histidine, L-lysine, and Nα-acetyl-L-lysine were labeled by both routes. Equivalent products resulted. (i) A stoichiometric amount of the metal chloride was added to a 3 mM solution of 1-(p-benzenediazonium)-ethylenedinitrilotetraacetic acid in 0.8 M HCl at 4°, the resulting solution was neutralized with solid $NaHCO_3$, added to an equivalent quantity of the acetylated amino acid, and maintained at 4°, pH 8 for 2 hr. (ii) A stoichiometric amount of 1-(p-benzenediazonium)-ethylenedinitrilotetraacetic acid in 0.8 M HCl at 4° was added to an ethanol solution of the acetylated amino acid, the reaction mixture was neutralized with excess solid $NaHCO_3$, and the mixture was stirred at 4°, pH 8.3 for 2 hours. The pH was then lowered to remove excess bicarbonate, and the desired metal chloride was added to the solution.

Attachment of chelates or chelating agents to human serum albumin was accomplished by adding a tenfold molar excess of the diazonium reagent to 0.5 mM albumin in 0.15 M NaCl at 4°. The reaction mixture was stirred for several hours at 4°, with the pH maintained at 8 by additions of 1 M NaOH. The modified protein was separated from hydrolyzed reagent either by dialysis or by a 1 x 15 cm Bio-gel P-100 column. Labeling yield was determined by scintillation counting and titration with indium; chelating groups were found to be attached mainly to lysine and histidine residues (Leung and Meares, in preparation). Lanthanide ions were found to bind specifically to the protein-bound EDTA groups when the modified albumin was dissolved in 0.1 M citrate, pH 6.5.

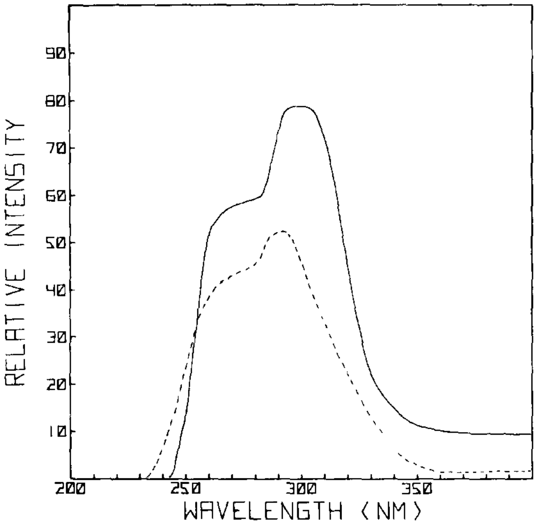

Figure 2. The fluorescence excitation spectra of albumin-bound terbium (III), (---), λ_{em} = 545 nm, concentration 3.8 μM; and europium (III), (——), λ_{em} = 618 nm, concentration 28 μM,both in 0.10 M Na-citrate, pH 6.5.

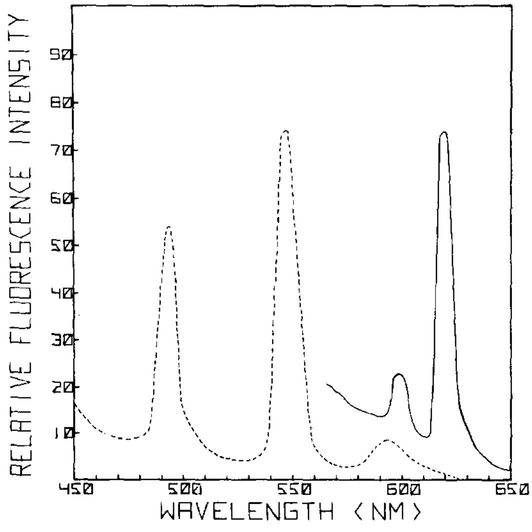

Figure 3. The fluorescence emission spectra of albumin-bound terbium (III), (---), λ_{ex} = 300 nm, concentration 3.8 μM; and europium (III), (——), λ_{ex} = 310 nm, concentration 28 μM, both in 0.10 M Na-citrate, pH 6.5.

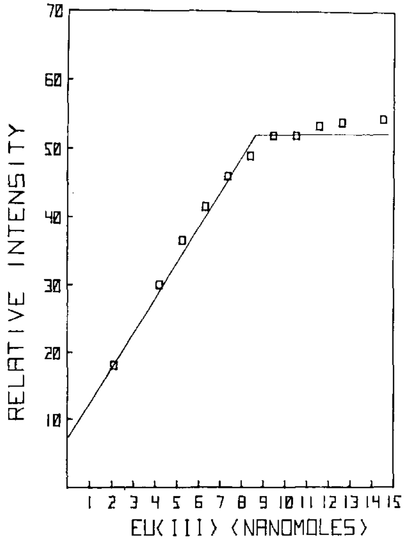

Figure 4. Fluorescence titration of albumin-bound EDTA groups (9 nanomol) with Eu(III) in 0.1 M Na-citrate, pH 6.5. Fluorescence of Eu(III) excited at 310 nm and monitored at 618 nm.

Fluorescence measurements were made at room temperature, using a Perkin-Elmer MPF-2A instrument operated in the ratio mode. Spectra were not corrected for instrumental response.

RESULTS: The fluorescence excitation and emission spectra of albumin-bound terbium (III) and europium (III) chelates are shown in Figs 2 and 3. The lanthanide emission spectra are very similar to Fig 3 for all compounds studied, but the fluorescence intensity is much greater for the albumin conjugates than for simple chelates.

The lanthanide excitation spectra depend markedly on the nature of the group attached to the EDTA moiety. Fig 2 shows that the excitation spectra of the albumin conjugates are qualitatively similar; those of the amino-acid conjugates are also similar. This is evidently due to efficient energy transfer from the aromatic ring which is adjacent to the EDTA group (see Fig 1), since the simple EDTA chelate of Tb (III) shows a single excitation peak at 240 nm, while the EDTA chelate of Eu (III) has its largest excitation peak at 390 nm.

Fig 4 shows the results of a titration of albumin-bound EDTA groups with Eu (III), monitored by the increase in europium fluorescence at 618 nm. The observed endpoint agrees with that determined independently by titration with indium. A similar fluorescence titration was performed using Tb (III), with identical results.

DISCUSSION: The measurement of energy transfer is a powerful tool for the determination of donor-acceptor distances in the range 10-100 Å (1). However, the polarization of electronic transitions limits the usefulness of aromatic organic molecules for such studies (8-10). Lanthanide ions have been proposed as energy donors which do not exhibit significant polarization effects (13,14) and metal ions such as cobalt (II) have been suggested as unpolarized energy acceptors (11,12). We find that when bifunctional chelating agents are attached to human serum albumin, their terbium and europium chelates exhibit lanthanide fluorescence which can be easily observed and quantitated when the bound europium concentration is less than 10^{-5} M or when the bound terbium concentration is less than 10^{-7} M. For each metal, this represents an enhancement of 2 to 3 orders of magnitude over the fluorescence of the simple EDTA chelate. In addition, we have found that the chelate of cobalt (II) with 1-(p-aminophenyl)-EDTA exhibits broad absorption in the range 420 to 600 nm with $\epsilon_{max} \simeq 10\ M^{-1}\ cm^{-1}$, similar to the behavior of other Co(II) chelates (18).

These results indicate that the bifunctional chelating agents can be used to prepare a new class of energy donors and acceptors which are less subject to uncertainties in K^2 than those reagents now in general use. Also, the sharp fluorescence lines of the lanthanides permit internal checks for polarization effects, since quenching of individual lines can be measured and examined for consistency.

The bifunctional chelating agents offer considerable versatility in choice of reactive group, since the aromatic amino group (Fig 1, R = NH_2) can be acylated, alkylated, diazotized, or otherwise modified to form products which will interact with biological systems. In addition, the bifunctional chelating agents can form metal complexes which possess a wide variety of useful spectroscopic and radioactive properties, permitting several different physical techniques to be applied with minimal changes in the system under study.

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