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MEASUREMENT OF DISTANCE BETWEEN FLUORESCENT AMINO ACID RESIDUES AND METAL ION BINDING SITES. QUANTITATION OF ENERGY TRANSFER BETWEEN TRYPTOPHAN AND TERBIUM(III) OR EUROPIUM(III) IN THERMOLYSIN

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SUMMARY: Quantitation of the sensitized visible emission of Tb(III) arising from a Förster-type dipole-dipole nonradiative energy transfer between Trp-186 and a Tb(III) ion bound at calcium site S(1) of thermolysin yields a measured energy transfer efficiency of 1.54×10^{-3}. Using an R_o estimate of 3.37 Å for the Trp → Tb(III) donor-acceptor pair yields an estimated separation of 9.9 Å, in good agreement with the 9.1 Å distance estimated from x-ray structural results. The much greater Trp → Eu(III) energy transfer efficiency of 0.42, obtained from the quenching of protein fluorescence, arises from the charge-transfer nature of the acceptor transition, which also accounts for the lack of significant sensitized emission from Eu(III).

INTRODUCTION

The replacement by trivalent lanthanide ions, Ln(III), of bound Ca(II) ions in proteins provides the molecular biologist with a choice of probe ions exhibiting a variety of useful spectroscopic and magnetic properties (1-4). The ability of certain members of this series, notably Eu(III) and Tb(III), to luminesce in fluid solution at room temperature is a property of particular promise (4-9). These ions can be caused to luminesce by direct excitation of metal ion levels using laser sources (5,7-10) or, in the case of Tb(III), by sensitization through fluorescent aromatic amino acids (phenylalanine, tyrosine, tryptophan) (4,6). In the latter case the aromatic amino acid absorbs UV light in the 250-300 nm wavelength range and transfers a small portion of this energy to a nearby Tb(III) ion. This phenomenon is quite general, having been observed to occur for 36 out of 40 proteins examined in a recent survey (6). The sensitization of Tb(III) luminescence is generally attributed to a nonradiative energy transfer process,

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but prior to this work and another study in this laboratory involving a parvalbumin isotype (11), it has not been subjected to experimental scrutiny.

The goals of the present study are to quantitate energy transfer of this type, to determine its mechanism, and to evaluate the utility of such measurements in the estimation of distances. The object of our study is thermolysin (EC 3.4.24.4), a structurally well-characterized (12,13) endoproteinase for which Ln(III) ion binding has been studied by x-ray crystallographic techniques (14). In the native state thermolysin binds a Zn(II) ion at the active site and four Ca(II) ions, which play a structural role, two at double site S(1), S(2) and two more at isolated sites S(3) and S(4) (15). Addition of one equivalent of Ln(III) ion to a solution of thermolysin quantitatively replaces the Ca(II) ion at site S(1) even in the presence of excess Ca(II) in solution (8,14,16). Although thermolysin contains several phenylalanine and tyrosine residues in addition to three tryptophans (12), it is possible selectively to excite the tryptophan fluorophore by irradiating the protein with light of 295 nm wavelength.

The present system is complicated by the presence of three potential energy donors: Trp-55, Trp-115, and Trp-186. Examination of the three-dimensional structure (13) reveals, however, that the centers of the three indole fluorophores are, respectively, 24.8, 20.2, and 9.1 Å from the Ln(III) ion binding site S(1). If a Förster-type dipole-dipole mechanism with an r^{-6} distance dependence accounts for the energy transfer, then Trp-55 and Trp-115 are far too distant to be involved significantly in this process when compared to Trp-186. Thus, while all three tryptophan moieties participate in protein absorption and fluorescence, only Trp-186 can be expected to participate in the Trp → Ln(III) ion energy transfer of interest.

EXPERIMENTAL SECTION

Thermolysin, obtained from the Sigma Chemical Corporation, was recrystallized as described elsewhere (17,18). Enzyme concentrations were determined by measuring the absorbance at 280 nm using $E^{280}_{1\%} = 17.65$ and a molecular weight

of 34,600 (13,19). The enzyme was assayed spectrophotometrically using furyl-acryloylglycyl-L-leucyl amide (20). Activities measured in a 0.05 M Tris buffer, pH 6.0, containing 0.1 M NaBr and 10 mM $CaCl_2$ at 25° yielded averaged $k_{obs}/[E]_o$ values of 12.1×10^5 min^{-1} M^{-1} and did not change upon the addition of Ln(III) ions. All luminescence experiments were carried out in 0.05 M Tris buffer, pH 6.0, containing 0.1 M NaBr and 1 mM $CaCl_2$ at 25° using a Perkin-Elmer MPF-44A spectrofluorimeter equipped with a differential corrected spectra microprocessor unit. The quantum yield of Trp in the protein was determined on this instrument using an aqueous solution of tryptophan as a standard (ϕ=0.13) (21). The quantum yield of Tb(III) bound to the protein was determined using the 488 nm line of an argon ion laser as the excitation source and an aqueous $TbCl_3$ solution as a standard (ϕ=0.065, the average of two reported values (22,23)). For this determination ε^{488} was estimated as 0.0688 M^{-1} cm^{-1} from model system studies. Visible absorption spectra of various Tb(III) chelate complexes were obtained using a Cary 118 spectrophotometer. The Ln(III) solutions were standardized using arsenazo as indicator (24). Absorption and emission spectra were digitized and spectral overlap integrals were calculated using a Mod-Comp II/25 computer system.

RESULTS AND DISCUSSION

Förster's theory of nonradiative energy transfer by a dipole-dipole mechanism (22,26) is well known and only the necessary equations will be given here. The measured efficiency of energy transfer, E, is related to the actual donor-acceptor separation, r, by eq. 1. R_o, the critical distance for 50% energy transfer, is given in eq. 2, where κ^2 is the orientation factor, and ϕ_{Trp} is the quantum yield

$$r = R_o[(1-E)/E]^{1/6} \qquad (1) \qquad R_o^6 = 8.78 \times 10^{-25} \kappa^2 \phi_{Trp} n^{-4} J \qquad (2)$$

of the donor tryptophan in the absence of acceptor. n is the refractive index of the medium intervening between the donor and acceptor. J is the spectral overlap integral defined in the caption of Figure 1.

Energy transfer efficiencies greater than a few percent can be measured from the effect of an energy acceptor on the luminescence intensity of the energy donor (eq. 3), where I and I_o are the emission intensities of tryptophan in the

$$E = [1-(I/I_o)] \qquad (3) \qquad E = \frac{A_{Tb(III)}}{A_{Trp}} \cdot \frac{\phi_{Trp}}{\phi_{Tb(III)}} \qquad (4)$$

presence or absence of acceptor, respectively. For very low efficiencies such as found for Tb(III) as an acceptor (vide infra), $I \simeq I_o$ and eq. 3 is useless. In

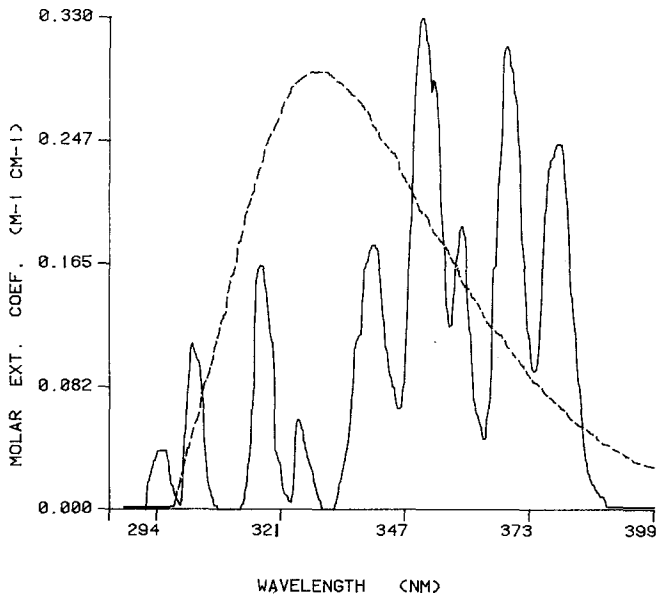

Figure 1. Plots of thermolysin fluorescence (λ_{ex} = 295 nm) on an arbitrary scale (dashed curve) and absorption spectrum of a 1:1 Tb(III) complex of diethylenetriaminepentaacetate (solid curve, scale on left hand ordinate) corrected for background ligand-based absorption. These data are used to evaluate the spectral overlap integrals, $J = \int F(\nu)\varepsilon(\nu)\nu^{-4}d\nu / \int F(\nu)d\nu$ where $F(\nu)$ is the fluorescence intensity of the energy donor, $\varepsilon(\nu)$ is the molar extinction coefficient of the energy acceptor (M^{-1} cm^{-1}), and ν is the frequency in cm^{-1}.

such cases E can be measured by comparing the number of photons emitted individually by the donor (Trp) and the acceptor (Tb(III)) and a knowledge of their respective quantum yields according to eq. 4, where $A_{Tb(III)}$ and A_{Trp} are the integrated areas of luminescence emission (on a cm^{-1} scale) of Tb(III) and the donor Trp in the protein, respectively, and $\phi_{Tb(III)}$ and ϕ_{Trp} are the respective quantum yields.

When a solution of thermolysin is titrated with a $TbCl_3$ solution while monitoring Trp fluorescence (λ_{ex} = 295 nm, λ_{em} = 333 nm), no effect on the Trp emission intensity is observed, indicating that the energy transfer efficiency is less than a few percent, although sensitized emission in the visible region of the spectrum characteristic of Tb(III) is readily observed. On the other hand,

an analogous titration with $EuCl_3$ causes the protein fluorescence to decrease to 0.86 of its initial value after one equivalent of Eu(III) has been added. Addition of more Eu(III) causes no further fluorescence quenching. This result corresponds to an E value of 0.42 for the Trp-186 → Eu(III) pair, if all of the energy is considered to be transferred from the closest Trp residue. Significantly there is virtually no Trp-sensitized emission in the visible region of the spectrum from the bound Eu(III) ion.

Energy transfer to Tb(III) can be quantitated via eq. 4. The experimental ratio of integrated emission intensities, $(A_{Tb(III)}/A_{Trp})$, is 3.91×10^{-3} (average of three determinations), the average quantum yield of Trp in thermolysin, ϕ_{Trp}, was determined to be 0.19 and $\phi_{Tb(III)}$ was estimated to be 0.48 (18). A_{Trp} is taken as 1/3 of the total protein emission area. These results lead to an E value of 1.54×10^{-3}.

In order to obtain distance estimates from the measured values of the energy transfer efficiencies, it is necessary to have numerical values for the various factors in eq. 2. For an energy donor such as Trp with a polarized emission dipole and an effectively isotropic acceptor, such as an Ln(III) ion, κ^2 must lie between 1/3 and 4/3 (27). Using the published atomic positional coordinates for thermolysin (13), calculations show, for a Trp-186 emission dipole lying in the plane of the indole fluorophore, that κ^2 lies in the range 0.34 to 0.71. Taking the indole emission dipole direction to be that calculated by Yeargers (28) results in a κ^2 value of 0.37 which is used in our analysis. n is taken as 1.35, a value intermediate between that of water and of organic molecules containing only first row atoms. Owing to the extremely weak absorption properties of Ln(III) ions, recourse was made to the use of model complexes to obtain the absorption spectra necessary to the evaluation of the spectral overlap integrals, J. Figure 1 shows the protein Trp emission superimposed on the absorption spectrum of a Tb(III) chelate complex. Using a series of model Tb(III) complex spectra, J values were obtained ranging from 0.62 to 1.10×10^{-19} cm^6 mol^{-1} with an average value of 0.81×10^{-19} cm^6 mol^{-1}. These results lead to R_o values in the range 3.22

to 3.55 Å, averaging 3.37 Å. Taking this average R_o value along with the measured Trp → Tb(III) energy transfer efficiency leads to a distance of separation, r, of 9.9 Å. Although this value is 0.8 Å (9%) longer than that estimated from the x-ray structural coordinates, it is in good agreement considering the large uncertainties in the measured or estimated quantities that went into this determination. If κ^2 is taken as its isotropic value (2/3), respective R_o and r values of 3.72 and 10.9 Å result. This gives an idea of the magnitude of uncertainty introduced into distance estimates by making this assumption.

The much larger energy transfer efficiency with Eu(III) as the acceptor ion requires some comment. Were the excited levels of the f^6 configuration of this ion involved in energy reception, an R_o value only slightly greater (∿3.8 Å) than that found for the Tb(III) case would apply, and significant sensitized emission in the visible region would be expected. The solution to this apparent paradox lies in the fact that Eu(III) complexes often exhibit relatively low-lying ligand to metal charge transfer transitions (29-32). Overlap of protein emission with a relatively intense charge transfer band accounts for the sizeable quenching of Trp fluorescence by this ion. No emission from an f^6 excited state is to be expected since the charge transfer transition correlates with an f^7 configuration. Although it is impractical to measure this charge transfer absorption for Eu(III) bound to a strongly absorbing Trp-containing protein, such spectra have been measured for Eu(III) bound to a parvalbumin which contains no Trp (11). In this case an R_o value of 9.95 Å is estimated which, when used in conjunction with the present measured Trp → Eu(III) transfer efficiency, yields an r of 10.5 Å in reasonable agreement with the Tb(III) result. It should be cautioned that ligand to Eu(III) charge transfer bands are quite variable both in position and intensity. Thus R_o estimates for the Trp → Eu(III) pair are likely to be considerably less certain than those for Tb(III) as the energy acceptor.

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