Lanthanide Ion Luminescence Probes. Measurement of Distance between Intrinsic Protein Fluorophores and Bound Metal Ions: Quantitation of Energy Transfer between Tryptophan and Terbium(III) or Europium(III) in the Calcium-Binding Protein Parvalbumin

William DeW. Horrocks, Jr.,* and William E. Collier

Contribution from the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received October 8, 1980*

Abstract: Nonradiative energy transfer between a fluorescent tryptophan (Trp) energy-donor and Tb(III) or Eu(III) energy-acceptor ions bound to the CD and EF calcium-binding sites of parvalbumin from codfish has been quantitated. Efficiencies of energy transfer of 5.32×10^{-4} and 0.50 for the individual Trp → Tb(III) and Trp → Eu(III) donor–acceptor pairs, respectively, were obtained. In both cases the measured efficiencies are in accord with a Förster-type dipole–dipole energy-transfer mechanism. Values of the critical distances for 50% energy transfer, R_0, of 3.35 Å for Tb(III) and 10.2 Å for Eu(III) as acceptor ions were obtained. The large value in the latter case is due to efficient overlap of tryptophan emission with a ligand to Eu(III) charge-transfer band. In the case of Tb(III) it was necessary to use absorption spectral data from model complexes to evaluate R_0. The donor–acceptor separations, r, estimated from the measured transfer efficiencies, of 11.8 and 10.2 Å for Tb(III) and Eu(III) as respective acceptors are in good agreement with the 11.6-Å distance estimated from X-ray structural results. The predominately charge-transfer nature of the Eu(III) ion absorption accounts for the fact that the luminescence emission of this ion is not significantly sensitized upon binding to the protein when compared to Tb(III). In the latter case f–f transitions alone are involved in the energy reception.

The trivalent lanthanide ions, Ln(III), are well established as replacement probes for Ca(II) in a variety of proteins.[1-4] Of the various probe properties exhibited by members of the Ln(III) ion series, the ability of ions of certain of these elements, notably Tb(III) and Eu(III), to luminescence in aqueous solution appears to be particularly useful.[4-8] Of these two ions, Tb(III) alone is significantly sensitized to luminesce when bound to a protein upon irradiation of the UV absorption bands of the aromatic amino acid residues (phenylalanine, tyrosine, tryptophan). In contrast, both Eu(III) and Tb(III) are significantly sensitized to luminesce upon binding to nucleic acids.[9] In a survey of 40 proteins, Brittain et al.[5] found 36 of them to exhibit sensitized emission in the visible region characteristic of bound Tb(III) upon addition of this ion to solutions of the various proteins. Thus the sensitization of Tb(III) luminescence is an important and general phenomenon for studying the binding of this probe ion to proteins.

While this phenomenon is generally attributed to a radiationless energy transfer, the exact mechanism of the transfer has not been subjected to experimental or theoretical scrutiny. Several possibilities exist. In cases where the hydroxyl oxygen of tyrosine is directly coordinated to Tb(III), a through-bond energy transfer can take place. This situation is undoubtedly the exception, and in all other cases a through-space mechanism must apply. The most obvious candidate is a dipole–dipole mechanism although a dipole–quadrupole mechanism has been suggested also.[10]

The present research sets out to quantitate the energy transfer between the intrinsic fluorescent protein residue, tryptophan, and protein-bound Tb(III) and Eu(III) ions. In particular our goals are as follows: (1) to establish the mechanism of nonradiative energy transfer involved; (2) to determine why Eu(III) luminescence is not significantly sensitized in comparison with Tb(III); and (3) to obtain Förster-type energy-transfer parameters and to assess the utility of Trp → Eu(III) or Trp → Tb(III) energy-transfer measurements as distance probes.

With regard to our third goal it should be noted that the utility of Förster-type nonradiative energy transfer has been established as a valid measure of distance through the study of model systems and proteins of known structure for the following classes of energy-transfer donor–acceptor pairs: organic fluorophore → organic chromophore;[11,12] organic fluorophore → transition metal ion;[13] Tb(III) ion → transition metal ion;[14,15] and Eu(III) or Tb(III) ion → other Ln(III) ions.[8,16,17] Energy-transfer distance measurements between an organic fluorescent probe (proflavin) and Pr(III), Nd(III), or Ho(III) which involve the visible region of the spectrum have also been reported.[18,19] This paper represents the first attempt to quantitate and exploit fluoresence energy transfer from an intrinsic amino acid residue of a protein to a bound metal ion.

To achieve our goals, we have chosen as a model system to study a parvalbumin from codfish (*Gadus callarius* L),[20,21] namely, component cod III which contains a single tryptophan at a known

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position in the amino acid sequence. The three-dimensional structure of a homologous parvalbumin from carp (carp III, p*I* = 4.25) has been determined,[22] and the replacement of Ca(II) by Tb(III) ions in the CD and EF calcium-binding sites has been studied by X-ray techniques.[23,24] Thus cod III represents a model system for which the spatial juxtaposition of the luminescent donor, tryptophan, and the acceptor ions, Tb(III) or Eu(III), is reasonably well established. Our approach is to determine whether our luminescence measurements, along with the assumption of Förster-type dipole–dipole energy transfer,[25,26] are consistent with the donor–acceptor separations estimated from the primary sequence and X-ray structural results.

Experimental Section

Materials. Sodium dihydrogen phosphate, EDTA, EGTA, calcium acetate, sodium chloride, and Tris were supplied by Fisher Scientific. The piperazine, cyclohexanediaminetetraacetic acid (CDTA), and diethylenetriaminepentaacetic acid (DPTA) were purchased from Sigma Chemical Co. The dipicolinic acid, tryptophan, and quinine sulfate dihydrate were obtained from Aldrich Chemical Co. The lanthanide ions were introduced as their chloride salts. $TbCl_3$ and $EuCl_3$ were from Research Chemical Corp. while $NdCl_3$, $GdCl_3$, $SmCl_3$, and $LuCl_3$ were from the Alfa Division of Ventron Chemical. The Sephadex G-75 was obtained from Pharmacia and the DEAE-52 (diethylaminoethyl cellulose) was purchased from Whatman. Doubly distilled, deionized water was used throughout.

Instrumentation. Luminescence spectra were measured by using a Perkin-Elmer MPF-44A fluorescence spectrophotometer equipped with a microprocessor corrected spectra unit. Absorption spectra were recorded with a Cary Model 118-C scanning double-beam spectrophotometer. Absorbance measurements at single wavelengths were made with a Beckman-Guilford single-beam spectrophotometer.

The Eu(III) laser-induced luminescence excitation experiments were done with an apparatus described in detail elsewhere[27] whose major components include: a Molectron Model UV-100 pulsed nitrogen laser, a grating-tunable dye laser (Molectron Model DL-200) (for excitation of a sample held in a 1-cm cuvette). Luminescence emissions were collected at 90° and passed through a (J-Y Optical, Model 20) 0.2-m, *f*/3.5 monochrometer and detected, after passing through appropriate cutoff filters by a Hamamatsu R928 photomultiplier tube. After suitable amplification and conditioning to avoid overload problems due to scattered light the signal was fed into a Princeton Applied Research "boxcar" signal averager which then presents the excitation profile to an X-Y recorder as the laser wavelength is scanned through the transition region. In the present case a laser pulse rate of 10 Hz was used and the $^7F_0 \rightarrow {}^5D_0$ transition in the 578–580-nm region was excited while monitoring the $^5D_0 \rightarrow {}^7F_2$ emission at 612 nm. Rhodamine 6-G laser dye was used.

Preparation of Parvalbumin. The method of Bhushana Rao et al.[20] was followed with some modification. Codfish was obtained in the form of fresh frozen fillets in a local supermarket. The fillets contained no myoglobin-rich red muscle, and 500 g of muscle was used in the procedure. The muscle was extracted with 1000 mL of 10 mM Tris, 2 mM EDTA, and 2% glycerol at pH 8.70 in an Osterizer high-speed blender. This and all subsequent operations were carried out at 4 °C either in a cold room or in an ice bath. After blending for 1 min at maximum speed, the resultant homogenate was stirred in the cold room for 1 h. The homogenate was then centrifuged in a Sorvall RC-5 superspeed refrigerated centrifuge for 30 min at 10000 rpm. The pellet was discarded. The supernatant was fractionated by using ice-cold acetone. The acetone was added drop by drop from a separatory funnel to the supernatant while stirring in an ice bath. Addition of acetone was stopped at 45% acetone (v/v), and the resultant solution and precipitate were centrifuged for 75 min at 10000 rpm. The pellet was discarded, and enough cold acetone was added rapidly to the supernatant to make the solution 80% in acetone (v/v). The resulting solution and precipitate were again centrifuged for 75 min at 10000 rpm. To the supernatant was added excess cold acetone; however, no additional precipitation was observed. The pellet obtained in the last precipitation step was redissolved in a minimum volume of 20 mM Na_2HPO_4 and 24 mM NaCl, pH 7.30.

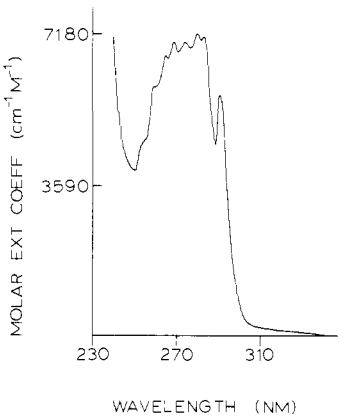

Figure 1. UV spectrum of cod III parvalbumin in 0.0162 M piperazine buffer, pH 5.70.

Virtually all of the pellet dissolved. The small amount of solid matter left was filtered out by using glass wool. The resulting solution contained the parvalbumin and was loaded onto a gel filtration column of Sephadex G-75 which had previously been equilibrated with 20 mM Na_2HPO_4 and 24 mM NaCl, pH 7.30. The column (60 × 2.5 cm, 1 L bed volume) was eluted with the equilibrating buffer at a flow rate of 2.5 mL/min. The fraction volumes were 10 mL. The absorbancies of the fractions at 280 nm were measured, and three peaks were observed on the resulting chromatogram. The first peak corresponds to high molecular weight proteins in the excluded volume of the column, the second peak represents the parvalbumins, and the third peak represents the residual acetone left from the fractionation. The fractions corresponding to the second peak were pooled, lyophilized, and dialyzed against 0.0162 M piperazine buffer at pH 5.70. After dialysis, this parvalbumin-containing solution was put onto a second column which consisted of DEAE-52 equilibrated with 0.0162 M piperazine, pH 5.70. The column (60 × 1.25 cm, 250-mL bed volume) was eluted with a linear NaCl gradient (450 mL × 450 mL, 0.0–0.10 M NaCl) at a flow rate of 0.40 mL/min. The fraction volumes were 4 mL. The absorbancies were measured at 259 and 280 nm. The resulting chromatogram revealed three peaks, the second and third of which correspond to the two major cod parvalbumin components. The second peak, which exhibits absorbance at 280 nm, is isotype cod III. The third peak with minimal absorption at 280 nm is isotype cod II which contains 10 phenylalanine residues but no tryosine or tryptophan. Fractions corresponding to the second peak were pooled, lyophilized, and dialyzed against 0.0162 M piperazine (pH 5.70) and reapplied to the same DEAE-52 column and eluted under the same conditions. The resulting chromatogram shows no cod II isotype to be present. The cod III fractions were pooled, and native polyacrylamide gel electrophoresis confirmed the homogeneity of this sample.

The UV absorption spectrum (Figure 1) shows absorptions due to phenylalanine, tyrosine, and tryptophan residues and is identical with that reported[21] for whiting component IIIb. Whiting IIIb and cod III both contain 10 phenylalanine, 1 tyrosine, and 1 tryptophan residues.[21] Protein concentrations were determined from absorbancy measurements at 280 nm by using $\epsilon_{280} = 7180\ M^{-1}\ cm^{-1}$.[21]

Lanthanide Ion Titrations. These were carried out on the fluorimeter by using parvalbumin concentrations in the range 20–70 μM. These concentrations are low enough to avoid problems with the inner filter effect. The initial volume of the protein solution to be titrated was 1 mL. Ln(III) ion solutions were added in increments of 0.2 equiv in a volume of 2 μL with a digital pipette. The accuracy and reproducibility of the pipette was checked by weighing methods. The Ln(III) ion solutions were prepared from the chloride salts and standardized by an EDTA titration using arsenazo as indicator.

Results and Discussion

Eu(III) Ion Laser Excitation Spectroscopy. In order to establish the nature of Eu(III) binding to parvalbumin cod III, we employed the $^7F_0 \rightarrow {}^5D_0$ laser excitation technique developed in this laboratory[7] which was applied previously to thermolysin[7,17] and carp parvalbumin.[16] In this experiment the transition between the nondegenerate $^7F_0$ ground and $^5D_0$ excited states is monitored by scanning a tuneable laser through the transition region 578–580 nm and monitoring luminescence emission from the $^5D_0$ state. Owing to the nondegenerate nature of the two states involved, more than one excitation peak implies more than one Eu(III) ion environment. Figure 2 (upper trace) shows the excitation spectrum

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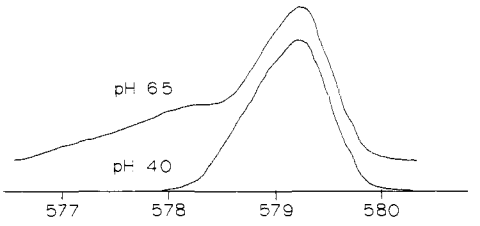

Figure 2. $^7F_0 \rightarrow {}^5D_0$ excitation spectra of cod III parvalbumin containing 2 equiv of Eu(III), λ_{em} = 612 nm: upper trace, pH 6.5; lower trace, pH 4.0.

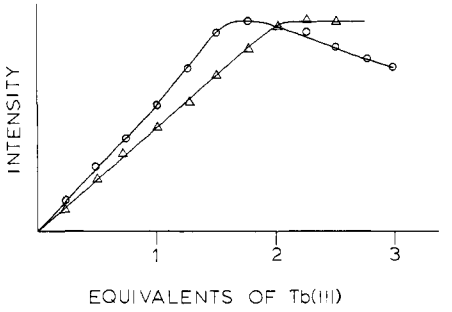

Figure 3. Plots of Tb(III) luminescence intensity (λ_{ex} = 295 nm; λ_{em} = 545 nm) as Tb(III) is added to cod III parvalbumin: open circles, pH 6.5; open triangles, pH 4.0.

of cod III to which 2 equiv of Eu(III) has been added at pH 6.5. As was the case with carp parvalbumin,[16] this signal can be resolved into three peaks, relatively sharp ones at lower energy due to occupancy of the CD and EF sites and a broader, higher energy feature due to a third, more solvent-exposed site. Figure 2 (lower trace) shows the result of lowering the pH to 4.0 where binding at the third site is eliminated, while occupancy of the CD and EF sites remains. This is, of course, the situation necessary for a straightforward analysis of our energy-transfer results, and our experiments were carried out under these low pH conditions.

Sensitized Emission from Tb(III). The Effect of pH. When cod III, to which 2 equiv of Tb(III) has been added, is irradiated with light of 295-nm wavelength, where only tryptophan absorbs strongly, luminescence in the visible region characteristic of emission from the $^5D_4$ excited state of Tb(III) is observed with the most intense peak at 545 nm. Luminescence titrations of cod III (λ_{ex} = 295 nm; λ_{em} = 545 nm) carried out as a function of added Tb(III) at pH 6.5 and pH 4.0 are shown in Figure 3. At pH 6.5 the luminescence titration curve shows a maximum at ~1.7 equiv of added Tb(III) and a fall off beyond this point, which is attributed to binding at a third site.[16,28] At pH 4, the pH of our energy-transfer experiments, no quenching effect is noted and the curve levels off at about 2.0 equiv of added Tb(III), consistent with the occupancy of both the CD and EF calcium sites by Tb(III) under these conditions.

Effect of Added Ln(III) Ions on Tryptophan Luminescence. Experiments were carried out wherein the luminescence of tryptophan (λ_{ex} = 295 nm; λ_{em} = 313 nm) was monitored as a function of added Ln(III) ions at pH 4.0. The results for Eu(III) and Tb(III) are shown in Figure 4. Eu(III) is seen to quench the protein luminescence quite effectively with only 25% of the initial luminescence remaining after 2 equiv of Eu(III) has been added. Addition of Tb(III) has virtually no effect on the protein luminescence. Similarly Gd(III), Sm(III), Nd(III), and Lu(III) cause no measurable changes in the protein luminescence when titrations (not shown) are carried out by using these ions.

Förster Theory. No attempt will be made here at a review of Förster's theory[25,26] with regard to distance measurements. Only the basic equations necessary to describe energy transfer by a dipole–dipole mechanism, needed for our analysis, will be presented. According to Förster, the efficiency of energy transfer, E, between a donor and acceptor is related to the actual distance

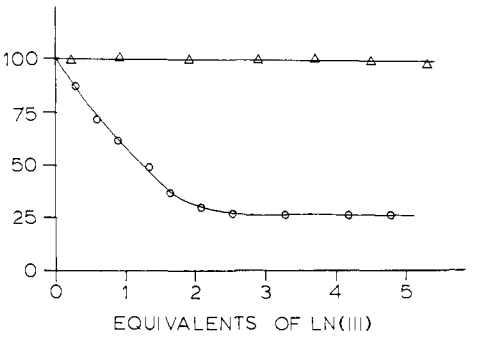

Figure 4. Plots of protein tryptophan fluorescence intensity (λ_{ex} = 295 nm; λ_{em} = 313 nm) as Ln(III) ions are added to cod III parvalbumin: open circles, Eu(III); open triangles, Tb(III).

of separation, r, and the critical distance for 50% energy transfer, R_0, by eq 1. R_0 is defined by eq 2, where κ^2 is the orientation

$$E = [1 + (r/R_0)^6]^{-1} \quad (1)$$

$$R_0^6 = 8.78 \times 10^{-25}\kappa^2\phi_{Trp}n^{-4}J \quad (2)$$

factor, ϕ_{Trp} is the quantum yield of the donor tryptophan in the absence of acceptor, and n is the refractive index of the medium intervening between the donor and acceptor. J is the spectral overlap integral defined by eq 3, where $F(\nu)$ is the luminescence

$$J = \frac{\int F(\nu)\epsilon(\nu)\nu^{-4}\,d\nu}{\int F(\nu)\,d\nu} \quad (3)$$

intensity of the donor, $\epsilon(\nu)$ is the molar extinction coefficient of the acceptor in units of M^{-1} cm^{-1}, and ν is the frequency in cm^{-1}.

Expressions for the Efficiency of Energy Transfer, in Terms of Experimental Observables. Two methods of measuring E were used in the present study. The first, applicable to tryptophan → Eu(III) energy transfer, depends on the fact that tryptophan emission is significantly quenched upon the binding of Eu(III) (Figure 5). In such a case, the observed transfer efficiency, E_{obsd}, is given by eq 4, where I and I_0 are the emission intensity of

$$E_{obsd} = [1 - (I/I_0)] \quad (4)$$

tryptophan in the presence and absence of acceptor (Eu(III)), respectively. For very low efficiencies such as found for Tb(III) (vide infra), $I \simeq I_0$ and eq 4 is useless. In such cases E can be measured by comparing the number of photons emitted individually by the donor (tryptophan) and acceptor (Tb(III)) and a knowledge of their respective quantum yields. Equation 5 applies in this case,[29,30] where $A_{Tb(III)}$ and A_{Trp} are the integrated areas

$$E_{obsd} = \frac{A_{Tb(III)}}{A_{Trp}}\frac{\phi_{Trp}}{\phi_{Tb(III)}} \quad (5)$$

of luminescence emission (on a cm^{-1} scale) of Tb(III) and tryptophan in the protein, respectively, and $\phi_{Tb(III)}$ and ϕ_{Trp} are the respective quantum yields. The quantum yield $\phi_{Tb(III)}$ is not necessarily a simple quantity as is discussed in a later section.

The present analysis is complicated by the fact that there is a single donor (Trp) but two Ln(III) acceptor ions. According to the X-ray results the two calcium ions lie precisely and fortuitously equidistant from the position of the tryptophan residue (vide infra). In cases of a single donor and two-equivalent, equidistant acceptors it can be shown that the observed efficiency, E_{obsd}, is related to that of an isolated donor–acceptor pair, E, by eq 6. For E_{obsd} values of less than a few percent, $E \simeq E_{obsd}/2$.

$$E = 1 - [1 - E_{obsd}]^{1/2} \quad (6)$$

Spectral Overlap Integrals, J. In order to carry out a quantitative analysis of the energy transfer results, we must evaluate

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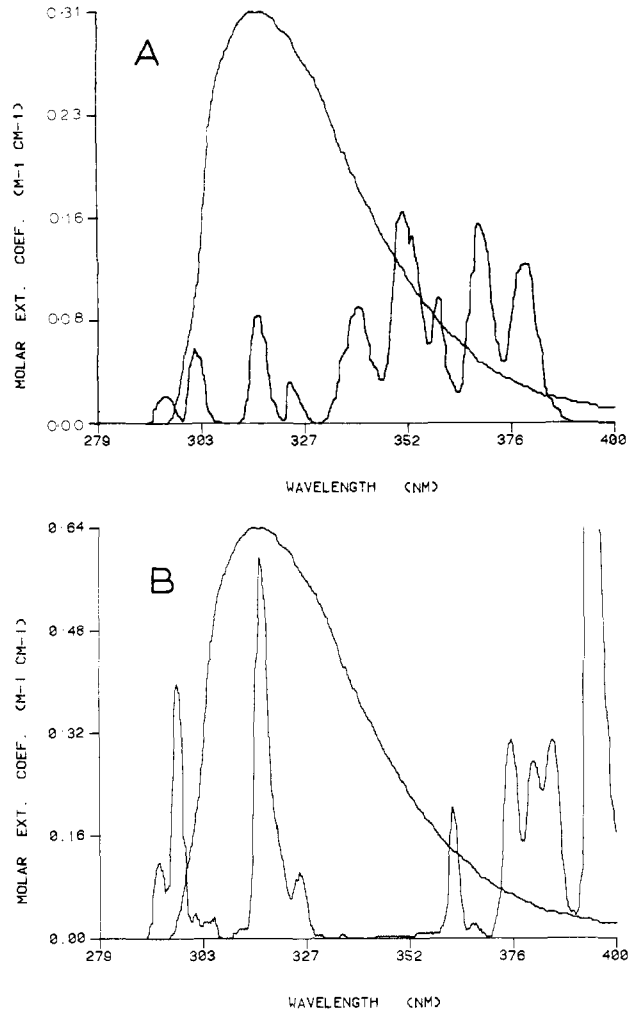

Figure 5. A: superpositions of tryptophan emission (λ_{ex} = 295 nm) from cod III parvalbumin (broad curve, λ_{max} = 313 nm) on absorption spectrum of a 1:1 Tb^{III}–DTPA complex. B: superposition of tryptophan emission (λ_{ex} = 295 nm) for cod III parvalbumin on the absorption spectrum of europium(III)–aqua ion.

the various factors making up R_0 (eq 2). The spectral overlap integral, J (eq 3), is perhaps the most important. In principle this quantity can be calculated from the luminescence emission spectrum of tryptophan in the protein and the absorption spectrum of a bound acceptor ion in the region of protein luminescence. The former quantity is readily available, however, the latter is unobtainable for f–f transitions owing to their low oscillator strengths. Recourse must be made to absorption spectra of model complexes. Figure 5A shows the absorption spectrum of a 1:1 Tb^{III}–DTPA complex superimposed on the protein emission spectrum. The relatively sharp, weak f–f transitions are evident. The absorption spectra of other terbium(III) aminopolycarboxylate model complexes are similar and lead to J values in the range (0.39–0.69) $\times 10^{-19}$ cm^6 mol^{-1}. In all cases the absorption spectra of the Ln(III) complexes were corrected for the presence of the long wavelength tail of a ligand-centered absorption band at higher energies. The absorption spectra of the corresponding Gd(III) complexes were used as a guide in this procedure. Figure 5B shows the absorption spectrum of the aquaeuropium(III) ion, so chosen because of the absence of a charge-transfer band in the spectral region of interest (vide infra), along with the emission spectrum of the protein. The J value calculated from these data is 0.99 $\times 10^{-19}$ cm^6 mol^{-1}.

Eu(III), with its f^6 electronic configuration, is known to exhibit an intense (relative to f–f transitions) charge-transfer band in the UV region of the spectrum in many of its complexes.[31-34] It was possible to obtain the difference absorption spectrum of carp III parvalbumin and a sample of this protein to which 2 equiv of Eu(III) was bound. The tail of the measured charge-transfer band overlaps significantly with the emission spectrum of tryptophan in cod III parvalbumin as shown in Figure 6. The absorption spectrum was recorded on carp III owing to the absence of tryptophan or tyrosine absorption which dominates the spectrum in the case of cod III. From the data shown in Figure 6 a J value of 353 $\times 10^{-19}$ cm^6 mol^{-1} was calculated.

Quantum Yields. The quantum yield of the emissive donor tryptophan, ϕ_{Trp} in the absence of energy transfer is needed for the evaluation of R_0 (eq 2) and in the calculation of the efficiency of energy transfer to Tb(III) (eq 5). This quantity was determined from the corrected emission spectrum of the protein excited at 295 nm by using as a standard an aqueous solution of the free amino acid tryptophan (ϕ = 0.13) at pH 7.[35] The quantum yield, ϕ_{Trp}, was found to be 0.16 for cod III parvalbumin.

The quantum yield of Tb(III), $\phi_{Tb(III)}$, bound to parvalbumin is more difficult to measure owing to the weak absorption properties of this ion. It was, however, found to be possible to measure this quantity directly for the $^5D_4$ emissive level (488 nm) on a 0.8 mM solution of parvalbumin to which 1.5 equiv of Tb(III) was bound. Quinine sulfate in 0.05 M sulfuric acid was used as a standard ($\phi_{quinine}$ = 0.55),[36] and the absorptivity of the 488 nm transition was inferred from the absorption spectra of several model Tb(III) complexes which could be obtained at higher concentrations (averaging 6.02 $\times 10^{-2}$ M^{-1} cm^{-1}). This procedure yielded $\phi_{Tb(III)}^{488}$ = 0.30. A series of aminopolycarboxylate model complexes gave values for this quantity ranging from 0.16 to 0.33. It should be noted that the quantity $\phi_{Tb(III)}$ required by eq 5 is not $\phi_{Tb(III)}^{488}$, but the quantum yield for excitation into the acceptor levels. As can be seen in Figure 5A, the transition at 488 nm is not involved in energy reception. Calculations based on a series of model complex absorption spectra show that the transitions at the following wavelengths (nm) yield the fractional contributions to the overall spectral overlap integral J indicated in parentheses: λ = 304 (0.063), 318 (0.178), 326 (0.087), 341 (0.242), 352 (0.256), 359 (0.037), 369 (0.098), 380 (0.039), 488 nm (0.00). Thus it is transitions in the 300–370-nm region which correspond to the receptor levels of Tb(III). A comparison of the f–f absorption spectrum of a model complex with the corrected excitation spectrum of the same system (not shown), recorded at comparable spectral bandwidths, shows that the two spectra are virtually superimposable. This implies that the quantum yields for excitation into various bands, including the $^5D_4$ emissive level, are nearly identical. Only for the bands at 304 and 318 nm (which account for only 24% of the overlap) do the quantum yields appear to fall off appreciably. We account for this by using a reduction factor of 0.92 in calculating the effective quantum yield for Tb(III) for excitation into the acceptor levels. Similar results were obtained for the other model complexes studied. We take the measured quantum yield for Tb(III) in parvalbumin (0.30) at 488 nm, decreased by a factor of 0.92, to be a reasonable estimate of the quantum yield for excitation into the acceptor levels in the 300–370-nm region.

The relative constancy of quantum yield as a function of excitation wavelength is not surprising, although there is some controversy regarding this point in the literature.[37-41] Relaxation

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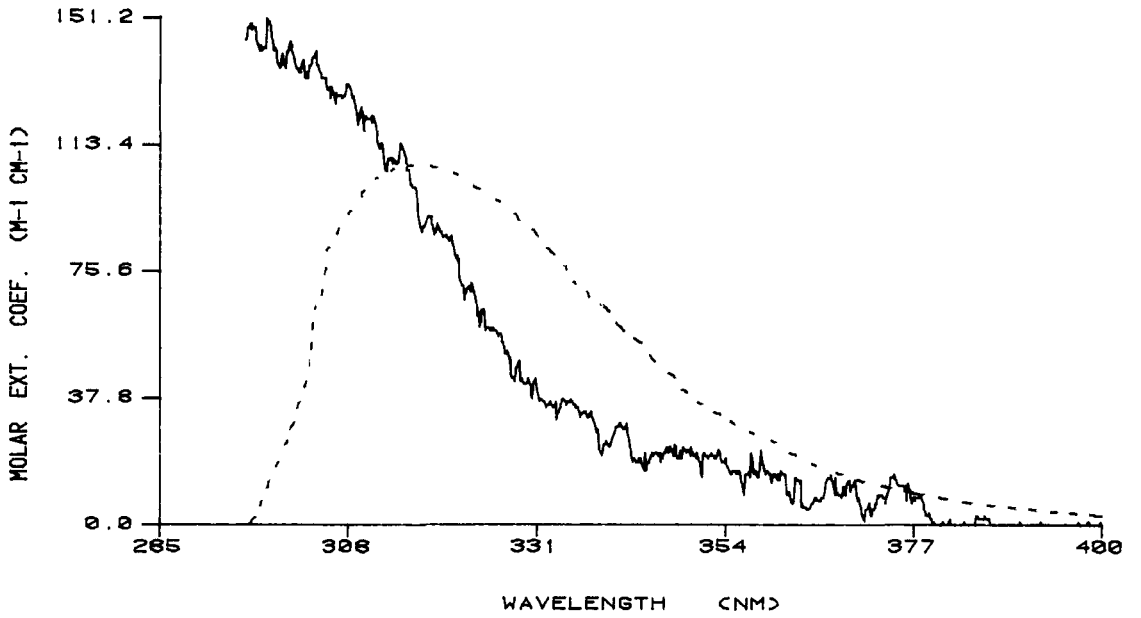

Figure 6. Difference absorption spectrum (charge-transfer band) of carp III parvalbumin with and without 2 equiv of Eu(III) (solid curve) and emission spectrum of tryptophan (λ_{ex} = 295 nm) in cod III parvalbumin (dashed curve).

from the higher excited states to the $^5D_4$ emissive level occurs in ~ 10 μs,[42] corresponding to a nonradiative rate constant of ~ 100 ms^{-1}. The most efficient nonradiative relaxation process available to the $^5D_4$ excited state of the terbium(III)–aqua ion involves energy transfer to the OH vibrational manifold of coordinated water molecules ($k_{OH} \simeq 2$ ms^{-1}).[6] Since vibrationally induced radiationless relaxation of the higher states is expected to be even less efficient, it is unlikely that a deexcitation pathway to the ground ^7F_J manifold exists which can compete effectively with relaxation to the $^5D_4$ emissive level. In the absence of such pathways the quantum yields of the higher levels will be identical with that of the emissive level.

R_0 Values. With use of the J values and quantum yields obtained as described above, R_0 values (eq 2) were calculated. The refractive index, n, is taken as 1.36, a value intermediate between that of water (1.33) and that of organic molecules containing only first-row atoms (1.39). Since there is little latitude in the choice of this parameter it will not be discussed further.

The orientation factor, κ^2, is taken as $^2/_3$, its value for an isotropic donor and an isotropic acceptor. Both Tb(III) and Eu(III) very likely correspond to nearly isotropic acceptors. The small ligand field splittings found in Ln(III) complexes will cause the various acceptor levels to remain nearly degenerate, leading to effectively isotropic absorption over the profiles of the narrow absorption bands. The indole fluorophore of the donor tryptophan, on the other hand, is expected to have emission polarized in the plane of the ring system.[43] For a polarized donor and an isotropic acceptor, κ^2 must lie between the extremes 1/3 and 4/3.[44] Any angular motion of the emitter which is rapid with respect to its excited state lifetime will cause κ^2 to approach the isotropic limit more closely.[44] In the present case it can be shown that the distance estimate would vary by ±1.4 Å (±12%) if the maximum or minimum extremes of κ^2 were taken. Since there are two acceptors in the present system, the likelihood that the mean value of κ^2 would reside at one of the extremes is even more remote.[45]

The R_0 values for the Trp → Tb(III) pair range from 3.24 to 3.57 Å. For Trp → Eu(III) energy transfer a J value of 3.75 Å is calculated when the europium(III)–aqua ion is used as a model for the absorption spectrum. In this case the only absorption bands involved correspond to the relatively weak f–f transitions. Inclusion of the charge-transfer band of Eu(III) bound to parvalbumin (Figure 6) in the calculation of the spectral overlap integral dramatically increases the Trp → Eu(III) R_0 value to 10.2 Å.

The Model. The ideal model system for study in order to establish the mechanism and to quantitate the energy transfer between a fluorescent tryptophan residue and bound Tb(III) or Eu(III) ions would be a protein with a single tryptophan and a single Ln(III) ion binding site whose spacial relationship is known from crystallographic studies. No such system exists. Only for the proteins thermolysin[46] and parvalbumin[23,24] (carp III, pI = 4.25) have crystallographic investigations of Ln(III) ion binding been carried out. Thermolysin has three tryptophan residues and, depending on conditions, can bind either one or three Ln(III) ions.[46] This metalloenzyme will be the subject of a separate study. On the other hand, parvalbumin from carp, whose complete three-dimensional structure is known,[22] contains two principal Ln(III) ion binding sites (the CD and EF calcium-binding sites) but no tryptophan. It is only one of a large number of homologous parvalbumin isotypes found in the fast skeletal muscles of vertebrates.[47-50] They all bind two calcium ions strongly and have very similar physical, chemical, and spectroscopic properties, implying the identity or near identity of their tertiary structures. Most parvalbumin isotypes contain many phenylalanine residues, but no tyrosine or tryptophan; however, two components are known which contain a single tryptophan, namely, isotypes whiting IIIb and cod III.[21] These proteins, both from the genus *Gadus*, are almost identical in amino acid composition, the only difference being that cod III has one less cysteine, one less valine, and one more serine residue than whiting IIIb. The single tryptophan can with some confidence, then, be ascribed to the same position in the primary sequences of the two proteins. The amino acid sequence has been determined for whiting IIIb.[51] Comparison of

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the sequences of whiting IIIb with carp III reveals that the tryptophan in the former, and by inference also in cod III, replaces phenylalanine-102 in the primary sequence of the latter.[50,52] With use of the published atomic coordinates[22] for carp III, the distances between the center of the phenyl ring of Phe-102 and the CD and EF metal ion binding sites were calculated. The phenyl ring was found to be fortuitously equidistant from *both* the CD and EF metal ion sites at a distance of 11.6 Å. The assumption that the center of the indole fluorophore is coincident with the center of the phenyl ring is an approximation leading to an uncertainty of about ±0.9 Å. It is this distance which will be compared with the results of our luminescence measurements.

It has been reported that under certain conditions crystals of parvalbumin can be grown which contain only a single Tb(III) ion at the EF site with no Tb(III) at the CD site.[23,24] The results of a recent NMR study of parvalbumin have been interpreted in terms of Yb(III) binding first at the EF site and subsequently at the CD site.[53] The present titration results (Figures 3 and 4) and the laser excitation experiments (Figure 2) show that under the conditions used by us the CD and EF sites are simultaneously occupied. Similar results have been obtained in this laboratory on carp parvalbumin.[16] Thus our model has a single energy donor (Trp) and two acceptor Ln(III) ions. For this reason the observed efficiency of energy transfer, E_{obsd}, must be converted to the efficiency of an isolated donor–acceptor pair (eq 6) for treatment according to Förster's equation.

Experimental Energy-Transfer Efficiencies. Of all the Ln(III) ions tested only Eu(III) caused significant quenching of protein tryptophan fluorescence upon binding to the protein. In this case the observed efficiency of energy transfer, E_{obsd}, is readily determined from the data (Figure 5) and found to be (eq 4) 0.75. This can be converted into the energy-transfer efficiency, E, for an isolated donor–acceptor pair (eq 6) of 0.50. It is noteworthy that while Trp → Eu(III) energy transfer is much greater than that observed for Trp → Tb(III), only Tb(III) is significantly sensitized to luminesce upon binding to the protein.

Energy-transfer efficiencies of less than a few percent cannot be measured accurately by monitoring donor luminescence in the presence and absence of an energy receptor. This situation applies in the case of the Trp → Tb(III) pair where energy transfer clearly occurs as evidenced by sensitized emission from Tb(III) upon irradiation at a wavelength where only tryptophan absorbs significantly. The binding of Tb(III) has, however, no measurable effect on the intensity of tryptophan fluorescence from cod III (Figure 4). Recourse must be made to an accurate measurement of the relative number of photons emitted by the donor and by the acceptor (assuming the latter is also luminescent) in order to quantitate energy-transfer efficiencies of less than a few percent. Equation 5 applies in this case.[29,30]

Taking $\phi_{Trp} = 0.16$ and $\phi_{Tb(III)} = 0.28$ along with the experimentally determined value for $(A_{Tb(III)}/A_{Trp})$ of 1.86×10^{-3}, one gets $E_{obsd} = 1.06 \times 10^{-3}$ and (eq 6) $E = 5.32 \times 10^{-4}$. This low value for the transfer efficiency is clearly too small to cause a measurable quenching effect on the tryptophan fluorescence; however it is large enough to cause an appreciable sensitization of the luminescence of bound Tb(III). Of the factors involved in the calculation of the energy-transfer efficiency (eq 5), $\phi_{Tb(III)}$ is the least certain since it depends on the use of the absorption spectral properties of model systems for Tb(III) bound to the protein. We estimate an uncertainty of ±20% in this quantity and hence in the values of E_{obsd} and E.

Distance Estimates. All of the factors which contribute to R_0 have uncertainties associated with them. As pointed out above in the discussion of the κ^2 parameter, even an uncertainty as great as a factor of 2 leads to uncertainties in the estimated distances of ±12% (±1.4 Å in the present example). This may be considered a reasonable upper limit to the uncertainty inherent in the present application of Förster-type energy-transfer distance measurements. Substantiation of the hypothesis that a dipole–dipole energy-transfer mechanism is operative in the present systems comes from the satisfactory agreement between distance estimates made from our measured energy-transfer efficiencies and calculated R_0 values and the actual distances estimated from the model. For the Trp → Tb(III) system, taking $E = 5.32 \times 10^{-4}$ and $R_0 = 3.35$ Å, we calculate a distance, r, of 11.8 Å in excellent agreement with the 11.6 Å estimated from the protein structure.

For the case of Eu(III) as an acceptor, values of E and R_0 of 0.50 and 10.2 Å, respectively, lead, of course, to an r value of 10.2 Å. This is shorter by 1.4 Å than the actual value for this acceptor ion. Although it was possible in this case to measure the absorption corresponding to the charge-transfer band of a protein-bound Eu(III), such measurements are difficult or impossible on tyrosine- or tryptophan-containing proteins. Furthermore, on the basis of model system studies, the position and intensity of the charge-transfer band depends in a sensitive manner on the details of the metal ion coordination environment. As an example, the J value calculated from the absorption spectrum of a Eu^{III}–EDTA complex and protein tryptophan emission is only one-fifth of that measured for Eu(III) bound to parvalbumin. Thus, the R_0 for Eu(III) is probably less reliable than the result for Tb(III) and can be expected to vary much more from system to system than the R_0 for Tb(III) as an acceptor.

Overall, it appears that both the sizeable energy-transfer efficiency from tryptophan to Eu(III) and the small value of this quantity with Tb(III) as the acceptor are adequately accounted for by a through-space, Förster-type dipole–dipole mechanism. The parameters established in the present study, particularly for tryptophan to Tb(III) energy transfer, should provide the basis for the interpretation of the results of future experiments on proteins of unknown structure to yield estimates of the distance between this intrinsic protein fluorophore and Ln(III) ion binding sites.

Quenching of Protein Fluorescence and Sensitized Emission by Ln(III) Ions. The very small energy-transfer efficiency for Trp → Tb(III) found in the present study is insufficient to cause measurable quenching of donor fluorescence; nevertheless readily detectable sensitization of Tb(III) luminescence is achieved. On the other hand with Eu(III) as the acceptor, energy transfer is quite efficient (50%), causing significant donor fluorescence quenching, but sensitization of Eu(III) emission is barely detectable. The explanation of this apparent paradox is that the principal acceptor transition in Eu(III) involves a ligand to metal charge-transfer transition which correlates with an f^7 excited-state configuration. No emission from f^6 excited states (e.g., $^5D_0$) is to be expected from such a level. It has further been suggested that the presence of charge-transfer states decreases the quantum yield of nearby f^6 excited states as well.[34] Thus, *while energy is efficiently transferred to Eu(III), it is virtually all radiationlessly dissipated and does not appear as luminescence.* The significant quenching of protein fluorescence upon binding of Eu(III) to a protein may find practical applications such as in monitoring the rate of Eu(III) ion binding in stopped-flow experiments.

In the case of Eu(III) bound to nucleic acids, the fluorescent donor 4-thiouridine has been implicated in energy transfer.[9] This fluorophore emits at energies much lower than tryptophan and well separated from any charge-transfer states. For nucleic acids both Eu(III) and Tb(III) exhibit strongly sensitized emission upon binding to the macromolecule.

Conclusions

Quantitation of the efficiency of energy transfer between the intrinsic fluorescent donor tryptophan and bound Ln(III) ions, large in the case of Eu(III) and small for Tb(III), is satisfactorily accounted for by a Förster-type dipole–dipole mechanism. The lack of significant tryptophan-mediated sensitization of Eu(III) luminescence upon binding to parvalbumin, and by inference also to other proteins, is attributed to the presence of charge-transfer acceptor levels having negligible quantum yields for luminescence. Parameters of Förster-type energy transfer have been established for the tryptophan → Tb(III) donor–acceptor pair which should allow the use of measurements of this type to establish distance

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relationships between intrinsic tryptophan fluorophores and metal ion binding sites in proteins.

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Communications to the Editor

Crystallographic Assessment of Absolute Configuration in 2′-Deoxyadenosine Cyclic 3′,5′-(R_p)-Phosphoranilidate. Direct ^{31}P-^{15}N Spin-Spin Coupling as a Probe for Configurational Assessment

Zbigniew J. Lešnikowski, Wojciech J. Stec,* and Wojciech S. Zieliński

Department of Bioorganic Chemistry
Centre of Molecular and Macromolecular Studies
Polish Academy of Sciences, 90-362 Łódź, Poland

Dorota Adamiak† and Wolfram Saenger

Department of Chemistry, Max-Planck-Institute for Experimental Medicine
D-3400 Göttingen, West Germany

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Recent works in this laboratory have shown that the diastereomeric nucleoside cyclic 3′,5′-phosphoranilidates and 3′,5′-phosphoranilidothioates are valuable intermediates in the stereospecific synthesis of P-chiral nucleoside cyclic 3′,5′-[^{18}O]-monophosphates,[1] cyclic 3′,5′-phosphorothioates,[2] and 3′,5′-phosphoroselenothioates,[3] which are important tools for mechanistic investigations of phosphotransferase reactions.[4] Because the PN → PX (X = ^{16}O, ^{18}O, S, Se) conversion proceeds with retention of configuration,[1,5] assignment of absolute configuration at the phosphorus atom in anilidates solves the problem of the absolute configuration of the corresponding nucleoside cyclic 3′,5′-[^{18}O]monophosphates, -phosphorothioates, and -phosphoroselenothioates.

Two criteria of configurational assessment at the phosphorus atom in nucleoside cyclic 3′,5′-phosphoranilidates were applied: (i) chemical-shift values in the ^{31}P NMR spectra of diastereomers,[2a,b] (ii) direct spin–spin coupling between phosphorus and exocyclic nitrogen-15 of the anilido moiety.[2d] Both criteria were adopted to nucleoside cyclic 3′,5′-phosphoranilidates from former works on diastereomeric 2-anilido-2-oxo-4-methyl-1,3,2-dioxaphosphorinanes[2d,6,7] and based on the assumption that the dioxaphosphorinanyl part of nucleoside cyclic 3′,5′-phosphoranilidates exists in the chair conformation. The results presented in this communication add proof to the validity of our assumption and deliver an additional example that direct spin–spin coupling constants between phosphorus and exocyclic nitrogen-15 nuclei can be applied as a certain criterion for distinguishing between axial and equatorial positions of the exocyclic arylamino group within the pair of diastereomeric 2-amido-2-oxo-1,3,2-dioxaphosphorinans. Both diastereomers of 2′-deoxyadenosine cyclic 3′,5′-phosphoranilidate (**1**) were obtained by reacting 2′-deoxyadenosine cyclic 3′,5′-phosphate (cdAMP) with triphenylphosphine–carbon tetrachloride–aniline.[2d] The reaction was performed in pyridine solution and both isomers of **1** were obtained in 46% yield. The products **1** were isolated and separated by chromatography on preparative TLC plates.[8] They were identical with authentic samples of **1** by using the criteria of TLC, mass spectrometry and ^{31}P NMR.[2b]

Compound (R_p)-**1** [R_f 0.27, ^{31}P NMR ($CHCl_3$, upfield from H_3PO_4) δ −2.88] crystallizes from methanolic solution in the orthorhombic space group $P2_12_12_1$ with cell dimensions a = 12.094 (3), b 22.409 (4), and c = 6.806 (2) Å; Z = 4. Intensities were recorded with an automatic Stoe four-circle diffractometer operated in the $\theta/2\theta$ scan mode (Ni-filtered, Cu Kα radiation) and corrected for Lorentz and polarization factors but not for absorption. The structure was solved by the multisolution method MULTAN[9] and refined by least-squares methods to the final disagreement index R = 7.09% including all 1550 reflections ($R = \sum||F_o| - |F_c||/\sum F_o$). On the basis of the ribose configuration we could choose the proper enantiomorph ($\bar{x}$, $\bar{y}$, $\bar{z}$) to establish the configuration around the phosphorus atom as R_p.

The structure of (R_p)-**1** is presented in Figure 1. The $O^{3'}$,-P,$O^{5'}$-dioxaphosphorinanyl ring of **1** exists in a chair conformation while the phenylamino group occupies the axial position. The bond lengths and the bond angles [P–NP 1.614 (7), NP–C_{10} 1.439 (9), NP–HNP 1.10 (7) Å; ∠P–NP–C_{10} 124.0 (5)°, ∠P–NP–HNP 119.0 (5)°, ∠C_{10}–NP–HNP 117.0 (6)°] indicate that hybridization of the phenylamino nitrogen atom is closer to sp^2.

† Institute of Chemistry, Adam Mickiewicz University, 60-780 Poznan, Grunwaldzka 6, Poland.

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