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Lanthanide Ion Luminescence Probes. Characterization of Metal Ion Binding Sites and Intermetal Energy Transfer Distance Measurements in Calcium-Binding Proteins. 1. Parvalbumin†

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ABSTRACT: Eu(III) laser excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition reveals spectral features characteristic of the occupation of the CD and EF Ca(II)-binding sites of parvalbumin by Eu(III) ions. In addition, at pH 6.5, a signal attributable to binding at a third site is observed. This feature is abolished upon lowering the pH to 3.8. At pH 6.5, the appearance of this feature correlates with the decrease in luminescence intensity during titrations of parvalbumin with Eu(III) or Tb(III) after more than ~1.8 equiv of either of these ions has been added. Eu(III) ions in the primary sites coordinate zero to two H_2O molecules while the third site involves about three coordinated H_2O molecules. Parvalbumins in which mixed pairs of lanthanide ions, Ln(III), occupy the CD and EF sites were prepared. Nonradiative energy transfer between Eu(III) and Tb(III) acting as luminescent donors and various other Ln(III) ions serving as acceptors was observed by monitoring the excited-state lifetimes of the donor ions using a pulsed dye laser apparatus. With the assumption of a Förster-type dipole–dipole mechanism, inter-binding-site distance estimates were made from our measurements and are in reasonable agreement with the distance (11.8 Å) obtained by X-ray crystallography, especially when Eu(III) is the donor. R_0 values (critical distances for 50% energy transfer) in H_2O solution range from 9.2 Å for the Tb(III)–Ho(III) donor–acceptor pair down to 5.7 Å for the Eu(III)–Ho(III) pair.

The use of trivalent lanthanide ions, Ln(III), as metal ion replacement probes in calcium-binding proteins is well established (Reuben, 1979; Ellis, 1977; Nieboer, 1975). The ability of certain Ln(III) ions, notably Tb(III) and Eu(III), to serve as luminescence probes has been noted (Horrocks & Sudnick, 1979a,b; Martin & Richardson, 1979). Of particular interest is the use of Tb(III) to Co(II) or Fe(III) Förster-type (Förster, 1948, 1965) energy transfer to measure distances between metal ion binding sites in proteins (Horrocks et al., 1975; Berner et al., 1975; Meares & Ledbetter, 1977). In this and the following paper (Snyder et al., 1981) we seek to establish inter-Ln(III)-ion energy transfer as a distance probe for use in calcium-binding proteins. Since there are a large number of proteins with two or more calcium-binding sites (Kretsinger, 1976; Kretsinger & Nelson, 1976), this class of probe holds the promise of considerable utility. The object of study in the present paper is a particular parvalbumin isotype from carp muscle (carp-III, pI = 4.25). Its primary sequence is known (Coffee & Bradshaw, 1973), and its complete three-dimensional X-ray structure has been determined to 1.85 Å resolution (Kretsinger & Nockolds, 1973). Furthermore, the coordination of Tb(III) to the two calcium-binding sites, denoted CD and EF, has been studied by X-ray techniques (Sowadsky et al., 1978). Parvalbumin thus provides a well-characterized model system on which to study inter-Ln(III)-ion energy transfer between the CD and EF sites which are separated by 11.8 Å in the native protein. In addition, using the Eu(III) ion excitation spectroscopic technique introduced by us earlier (Horrocks & Sudnick, 1979b), we are able to establish the details of Ln(III) ion binding to parvalbumin including the characterization of a third binding site.

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Our findings illuminate some earlier puzzles regarding Tb(III) binding as studied by luminescence techniques (Donato & Martin, 1974; Nelson et al., 1977). A preliminary account of this research has appeared (Horrocks et al., 1980).

Materials and Methods

Materials. Parvalbumin component III from carp was prepared according to the procedure of Pechere et al. (1971) with slight modifications. For the initial fractionation of low molecular weight proteins, acetone was used instead of ammonium sulfate as is described by Bhushana Rao et al. (1969). A DE-52 column was substituted for the DE-11 column in the final purification step.

All Ln(III) salts were obtained from either Research Organic/Inorganic or Alfa-Ventron Chemicals. Deuterium oxide (99.9%) was purchased from Sigma Chemical Co., and all other chemicals were reagent grade or the purest commercially available.

Methods. The protein concentration was determined by UV absorption using a molar extinction coefficient of 2000 M^{-1} cm^{-1} at the 259-nm absorption peak. Measurements were made with a Beckman-Guilford spectrometer (Model 252). The concentrations of Ln(III) solutions were determined by EDTA[1] titration employing arsenazo as indicator (Woyski & Harris, 1963).

The steady-state luminescence intensities of Tb(III)- or Eu(III)-parvalbumin complexes were measured on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Emission spectra of Tb(III)- or Eu(III)-parvalbumin complexes were also recorded on the same instrument, employing a corrected spectra microprocessor unit in the case of Tb(III).

Visible absorption spectra of $[Ln^{III}(DPA)_3]^{3-}$ complexes were recorded on a Cary 17 spectrophotometer, and circular dichroism spectra of parvalbumin were obtained on a Jasco spectrophotometer, Model L-20. Luminescence lifetimes of Tb(III)- or Eu(III)-parvalbumin complexes were measured by using a pulsed nitrogen laser pumped dye laser of which detailed descriptions may be found elsewhere (Sudnick, 1979).

Europium(III) excitation spectra for the $^7F_0 \rightarrow {}^5D_0$ transition were obtained by using the same pulsed dye laser equipped with a scan control unit. The laser (rhodamine 6G dye) whose spectral band width is ~0.01 nm was scanned from 577 to 580 nm while monitoring the $^5D_0 \rightarrow {}^7F_2$ emission at 612 nm. The emission intensity was recorded with the aid of a boxcar integrator (PAR-162).

A Perkin-Elmer (Model 310) curve resolver was used for analyzing the $^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu(III)-parvalbumin complexes. Overlap integrals between emission spectra of Tb(III)- or Eu(III)-parvalbumin complexes and absorption spectra of various model Ln(III) complexes were calculated on a Mod-Comp II/25 computer system.

Except as otherwise indicated, all experiments were performed with 0.1–1.0 mM solutions of parvalbumin (carp-III, p*I* = 4.25) in 0.01 M piperazine buffer. Hybrid Ln(III)-substituted parvalbumins for the energy-transfer measurements were prepared by adding 2 equiv of the appropriate 0.01 M stock Tb(III)–Ln(III) mixtures [Tb(III)/Ln(III) = 1:19 where Ln(III) = La(III), Nd(III), Ho(III), Er(III), or Pr(III)] or Eu(III)–Ln(III) mixtures [Eu(III)/Ln(III) = 1:19 where Ln(III) = Yb(III), Pr(III), or Nd(III)]. The pH was then lowered to 3.8 to displace Ln(III) ions from the third site on the protein (vide infra).

[1] Abbreviations used: DPA, 2,6-pyridinedicarboxylic acid; EDTA, ethylenediaminetetraacetic acid.

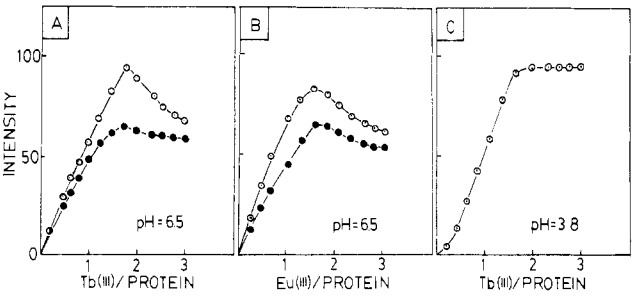

FIGURE 1: Ln(III) ion luminescence titration curves of parvalbumin. (A) 2.15×10^{-4} M parvalbumin (carp-I, p*I* = 4.47) titrated with Tb(III). Excitation at 259 nm (○) and 372 nm (●); luminescence emission monitored at 545 nm. (B) 1.19×10^{-4} parvalbumin (cod-II, p*I* = 4.1) titrated with Eu(III). Excitation was at 259 nm (○) and 395 nm (●), with monitoring of the emission at 614 nm. (C) 2.15×10^{-4} M parvalbumin (carp-I, p*I* = 4.47) titrated with Tb(III) at pH 3.8, excited at 259 nm and monitored at 545 nm.

Results

Luminescence Titrations of Parvalbumin with Tb(III) or Eu(III). The metal ion luminescence emission intensities exhibited by parvalbumin solutions as a function of added Tb(III) or Eu(III) at pH 6.5 are shown in parts A and B, respectively, of Figure 1. An intensity maximum is reached at a Ln(III)/protein ratio of ~1.8, beyond which there is a decrease, a result previously observed in the case of Tb(III) (Donato & Martin, 1974; Nelson et al., 1977). This result is obtained both by direct excitation of metal ion levels [λ_{ex} = 372 nm for Tb(III), 395 nm for Eu(III)] and by sensitization through phenylalanine (λ_{ex} = 259 nm). As shown in Figure 1C, the shape of the titration curve changes dramatically at lower pH. At pH 3.8 there is no dip in the emission intensity beyond 2 equiv of Tb(III). This finding is true for direct excitation at 372 nm and for Eu(III) as well. However, a further decrease (to pH 3.0) virtually abolishes Tb(III) emission. Circular dichroism measurements in the 210–250-nm region were carried out. The trough at 222 nm, which is diagnostic of α-helix content, remains unchanged in the pH range 6.5–3.7 but is virtually abolished at pH 3.0, suggesting protein denaturation at this low pH value.

Deuterium Isotope Effect on Shapes of Tb(III) and Eu(III) Titration Curves. Figure 2A shows the results of Tb(III) luminescence titrations carried out in both H_2O and D_2O solutions for direct excitation of the metal ion level (λ_{ex} = 372 nm). Identical behavior in H_2O and D_2O solutions is observed (Figure 2B) for Eu(III) (λ_{ex} = 395 nm). It should be noted that the quenching effect beyond 2 equiv of Ln(III) ion is not evident in the D_2O solutions. On the other hand, if the Tb(III) or Eu(III) is sensitized through the phenylalanine chromophores of the protein (Figure 2C,D), the characteristic quenching is observed following the intensity maximum at ~1.8 equiv of added Ln(III) ion for both H_2O and D_2O solutions.

Eu(III) Titration of Parvalbumin followed by $^7F_0 \rightarrow {}^5D_0$ Excitation Spectroscopy. The excitation spectrum of parvalbumin in an H_2O buffer at pH 6.5 to which 10 equiv of Eu(III) has been added is shown in Figure 3A. This spectrum can be resolved into three peaks: A, 579.2 nm; B, 579.6 nm; C, 578.4 nm. Peaks A and B are relatively sharp and appear first during the course of a titration of parvalbumin with Eu(III); they are attributed to Eu(III) ions occupying the CD and EF sites. Experiments on crystals of parvalbumin prepared so as to have Eu(III) in only the EF site (Sowadsky et al., 1978) show that peak B corresponds to Eu(III) in this site. A broad peak C, which becomes significant only after ~1 equiv of Eu(III) has been added, is attributed to Eu(III)

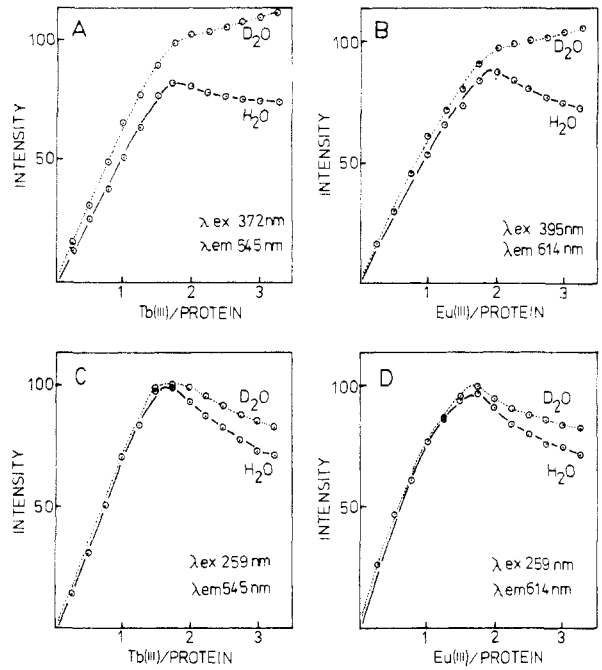

FIGURE 2: Deuterium isotope effect on luminescence titration curves of parvalbumin when Ln(III) ion is excited directly or sensitized through the protein. 1×10^{-4}–1.4×10^{-4} M parvalbumin (carp-I, pI = 4.47) in 0.01 M piperazine buffer at pH 6.5.

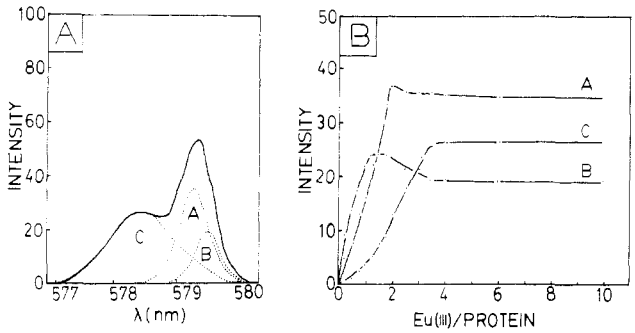

FIGURE 3: (A) $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of Eu(III)–parvalbumin (5.5×10^{-4} M carp-II, pI = 4.37, pH 6.5) and (B) titration curves showing change of each curve-resolved component of the spectrum during the course of titration.

binding at a third site. The behavior of the intensities of the various curve-resolved components of the excitation spectrum during the course of titration is shown in Figure 3B. When the same titration is carried out at pH 3.8, the broad peak C is absent and no intensity decrease is observed in the latter stages of the titration.

Determination of the Number of Water Molecules, q, Coordinated at Various Sites. At pH 3.8, where only the CD and EF sites are occupied, the reciprocal lifetimes, τ^{-1}, were obtained on a 1:19 Eu(III)–Lu(III) hybrid at four different Eu(III) excitation wavelengths from 579.2 to 579.8 nm in pure H_2O ($\tau_{H_2O}^{-1}$) and also in solutions 50.0% and 66.7% in D_2O. The value of the reciprocal lifetime in pure D_2O solution was determined by linear extrapolation to 100% D_2O (Horrocks et al., 1977). The τ^{-1} results were, within experimental error, constant across the profile of the excitation spectrum (peaks A and B of Figure 3A) yielding $\tau_{H_2O} = 1.81$ ms^{-1} and $\tau_{D_2O} = 0.65$ ms^{-1}. By use of eq 1 (Horrocks & Sudnick, 1979a,b)

$$q = 1.05(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1}) \quad (1)$$

the number of coordinated water molecules was found to be 1.2 with an estimated uncertainty of ±0.5.

Measurement of the reciprocal lifetimes corresponding to excitation of the Eu(III) bound at the third site (peak C,

Table I: Reciprocal Excited-State Lifetimes, τ^{-1}, Energy-Transfer Efficiencies, E, Spectral Overlap Integrals, J, Critical Distances for 50% Energy Transfer, R_0, and Experimentally Estimated Distances, r, between CD and EF Sites of Parvalbumin for Various Donor–Acceptor Ln(III) Ion Pairs[a]

donor	acceptor	τ^{-1} (ms^{-1})	E[b]	J ($\times 10^{18}$ cm^6 mol^{-1})[c]	R_0 (Å)[d]	r (Å)[e]
Eu(III)	*f*	1.81				
Eu(III)	Pr(III)	1.98	0.076	6.87	8.3	12.6
Eu(III)	Nd(III)	2.24	0.183	10.8	9.0	11.5
Tb(III)	*g*	0.78				
Tb(III)	Nd(III)	1.69	0.538	7.45	9.0	8.8
Tb(III)	Ho(III)	1.22	0.361	8.48	9.2	10.1
Tb(III)	Er(III)	1.11	0.297	3.27	7.9	9.1
Tb(III)	Pr(III)	0.82	0.207	2.42	7.5	9.4

[a] All symbols are as defined in Horrocks et al. (1980). [b] $E = 1 - (\tau/\tau_0)$. [c] $J = \int F(\nu)\epsilon(\nu)\nu^{-4}\,d\nu / \int F(\nu)\,d\nu$; calculated by using the absorption spectra of $[Ln^{III}(DPA)_3]^{3-}$ complexes. [d] $R_0^6 = (8.78 \times 10^{-25})\kappa^2\phi n^{-4}J$ with $\kappa^2 = {}^2/_3$, $n^{-4} = 0.294$, $\phi_{Eu} = 0.28$, and $\phi_{Tb} = 0.42$. [e] $r = R_0[(1-E)/E]^{1/6}$. [f] Averaged result for Gd(III), Yb(III), and Lu(III) nonacceptor ions. [g] Averaged result for La(III), Gd(III), and Lu(III) nonacceptor ions.

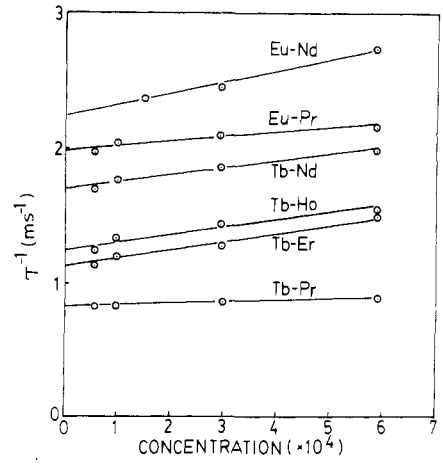

FIGURE 4: Plots of reciprocal excited-state lifetimes, τ^{-1}, of Eu(III) or Tb(III) energy donor ions with various acceptor ions vs. parvalbumin concentration. These experiments were carried out at donor ion to acceptor ion ratios of 1:19 at pH 3.8.

Figure 3A), $\lambda_{ex} = 578.0$ nm, by the same procedure yielded the following results: $\tau_{H_2O}^{-1} = 3.42$ ms^{-1}; $\tau_{D_2O}^{-1} = 0.86$ ms^{-1}. By use of eq 1 these findings suggest that $q = 2.7$ for this third site.

Energy Transfer from Tb(III) or Eu(III) to Other Ln(III) Ions in Parvalbumin. The reciprocal excited-state lifetimes of Eu(III) and Tb(III) in the various hybrid-substituted parvalbumins prepared as described earlier at pH 3.8 are given in Table I. The values reported were obtained by a linear extrapolation of the data to zero protein concentration (Figure 4). This procedure eliminates the contribution from any intermolecular collisional quenching mechanism (Thomas et al., 1978; Yeh & Meares, 1980). Since neither the high acceptor to donor ratio nor the extrapolation procedure was used in our preliminary report (Horrocks et al., 1980), the present values are slightly different and should be considered to supercede those given earlier. The τ^{-1} values for a nonacceptor ion [La(III), Gd(III), Yb(III), or Lu(III)] in the other site are also given and are taken to be the values, τ_0^{-1}, in the absence of Förster-type energy transfer. The efficiencies of energy transfer, E, calculated from these results are also given in Table I. It should be noted that because of the 1:19 ratio of donor (D) to acceptor (A) ions in the hybrids, a statistical distribution results in the following occupation of the CD and EF sites: (A_{CD}, A_{EF}) 90.3%; (A_{CD}, D_{EF}) 4.75%;

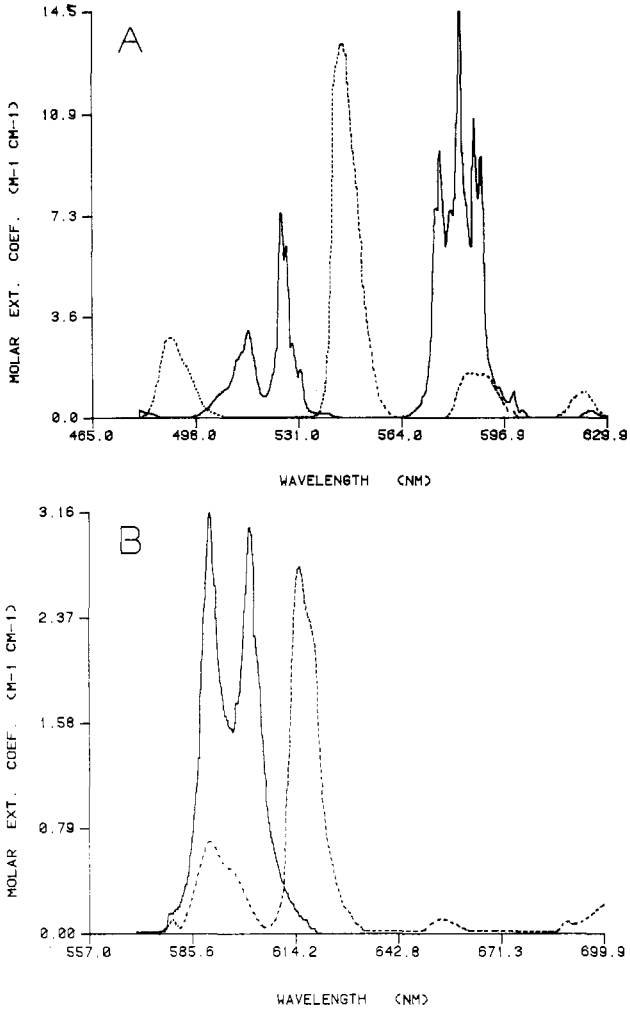

FIGURE 5: (A) Corrected emission spectrum of Tb(III) bound to parvalbumin (dashed line) and absorption spectrum of $[Nd^{III}(DPA)_3]^{3-}$ (solid line). (B) Emission spectrum of Eu(III) bound to parvalbumin (dashed line) and absorption spectrum of $[Pr^{III}(DPA)_3]^{3-}$ (solid line). The emission spectra are on arbitrary scales.

(D_{CD}, A_{EF}) 4.75%; (D_{CD}, D_{EF}) 0.25%. The 90.3% of parvalbumin molecules with acceptor pairs are spectroscopically silent while the 0.25% with donor pairs are present at low enough concentration so as not to affect the results which arise from the total of 9.5% of the molecules which contain donor–acceptor pairs.

Emission and Absorption Spectra. For calculation of the spectral overlap integrals, J, the emission spectra of parvalbumin-bound Tb(III) and Eu(III) are required (Figure 5). Also needed are the absorption spectra of the various acceptor ions in a protein environment. Owing to the extemely low molar extinction coefficients of the Ln(III) ions, their spectra are unobtainable at attainable protein concentrations. Recourse was made to the absorption spectra of model complexes such as those of $[Ln(III)(DPA)_3]^{3-}$ complexes of Nd(III) and Pr(III) shown in parts A and B, respectively, of Figure 5. The absorption spectra of the corresponding Ho(III) and Er(III) complexes can be found in the following paper (Snyder et al., 1981).

Discussion and Analysis

Characterization of CD and EF Binding Sites. The Eu(III) $^7F_0 \rightarrow {}^5D_0$ excitation spectrum taken at pH 3.8 (not shown) can be curve resolved into two closely spaced (579.2- and 579.6-nm) Lorentzian components (peaks A and B of Figure 3A) attributable to Eu(III) ions occupying the primary CD and EF sites, respectively. The spectral feature attributable to occupation of a third site is absent at this low pH value. The excited-state lifetimes measured in either H_2O or D_2O solution do not vary appreciably as the excitation wavelength is stepped across the spectral profile. These averaged reciprocal lifetime results imply that $\sim 1.2 \pm 0.5$ water molecules are coordinated at each of these sites. This result is somewhat uncertain owing to the considerable overlap between the signals attributed to the individual sites. Deconvolution of separate exponential luminescence decays is only possible if they are well separated in the time domain, which appears not to the case here. Some comment is required, since the X-ray results (Kretsinger & Nockholds, 1973) on the native Ca(II) protein show that no water molecules are liganded to the Ca(II) ion in the CD site while one water molecule is coordinated at the EF site (the so-called solvent-accessible site). Since any OH oscillator in the first coordination sphere of Eu(III) will contribute to radiationless deexcitation of the $^5D_0$ state, it should be anticipated that the hydroxyl group of Ser-55, one of the ligands in the CD site, will contribute such an oscillator which would be expected to behave in this regard approximately as a half H_2O. Contributions from this source can be expected whenever our method is applied to a system with D_2O-exchangeable XH oscillators in the first coordination sphere of a Ln(III) ion (X = O, N, S, P, etc.). In practice, since only oxygen donor ligands are known to coordinate Ca(II) in proteins, only the hydroxyl moiety of serine or threonine will cause this complication. When all of the above is taken into consideration, an interpretation consistent with our data to within experimental error is as follows: the Eu(III) ion occupying the CD site possesses zero or, more likely, one coordinated water molecule, plus the serine hydroxyl group, while the EF site has one or, less likely, two coordinated water molecules. It is possible that one or both of the Ca(II) sites have increased their coordination numbers by adding an additional water molecule upon substitution of Ca(II) by Eu(III). There is precedent for just such a coordination sphere expansion in the case of Ca site 4 in thermolysin (Matthews & Weaver, 1974).

Binding of Ln(III) Ions at a Third Site. Before attempting to analyze the principal results of this research regarding the inter-Ln(III)-ion energy transfer, it is necessary to establish the details of the metal ion binding. Earlier experiments wherein Tb(III) luminescence was sensitized through the phenylalanine chromophores showed a maximum in the Tb(III) emission which occurred at somewhere between 1 and 2 equiv of added Tb(III) (Donato & Martin, 1974; Nelson et al., 1977), beyond which there was a decrease in luminescence intensity, hereafter referred to as quenching. These results are confirmed here where it is also shown that Eu(III) behaves analogously (Figure 2B–D), although in the case of Eu(III) the magnitude of the sensitization is smaller by a factor of ~ 100. Donato & Martin (1974) and Nelson et al. (1977) proposed that this quenching occurs because of Tb(III) binding to an additional site or sites on the protein. Our Eu(III) excitation spectroscopic experiments at pH 6.5 (Figure 3, peak C) provide definitive evidence for such an additional binding site; moreover, the occupation of this site correlates with the quenching of luminescence from peaks A and B during the course of a titration (Figure 3B). The intensity of component C (Figure 3B) attains a constant value before 4 total equiv of Eu(III) has been added to the protein. This implies that peak C arises from the binding of only a single Eu(III) ion. The quenching effect observed in the presence of more than 2 equiv of Tb(III) is abolished upon lowering the pH to 3.8 (Figure 1C), suggesting that the third binding site is eliminated

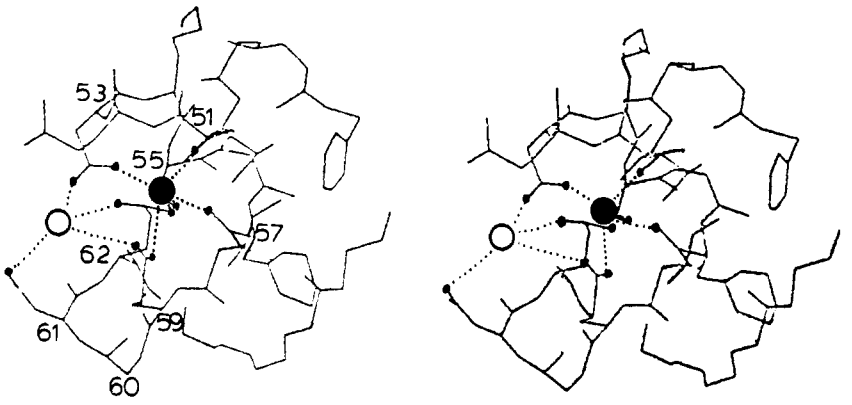

FIGURE 6: Stereo view of CD site and postulated third site of Ln(III)–parvalbumin (carp-III, pI = 4.25). The closed circle represents a Ln(III) ion in the CD site and the open circle represents a Ln(III) in the third site [adapted from Feldmann (1976)].

as the pH is lowered. This idea is corroborated by the Eu(III) excitation spectra recorded at pH 3.8 (not shown) wherein the peak attributed to the third site is absent and no quenching of the intensities of components A and B is observed during the course of the titration.

Two questions remain: What is the nature of the third binding site and what is the mechanism of the quenching? From the shape of the pH titration curve (not shown) it can be inferred that a ligand or ligands for the metal in the third site have a pK_a of ~4.5. This, not unexpectedly, implies the involvement of a carboxyl group or groups as ligands for the third site. The deuterium isotope effect on the reciprocal lifetime of Eu(III) bound to the site implies that about three water molecules are coordinated to this ion. With the assumption of a likely total coordination number of seven or eight for a Ln(III) ion bound to a protein, this result suggests that the protein itself supplies four or five ligands to the metal ion in the third site. Moreover, the relatively tight binding of Eu(III) to this third site, implied by the excitation titration curve (Figure 3C), would not be expected were the protein to supply fewer than three or four ligands to the metal.

Since binding of a Ln(III) ion produces a marked quenching effect on the same ion in the CD and EF sites, it is likely that the third site is in close proximity to one or both of these primary sites. Inspection of the primary sequence (Coffee & Bradshaw, 1973) and the atomic coordinates in three dimensions (Kretsinger & Nockholds, 1973) reveals that there is a total of 11 aspartic and glutamic acid residues in carp-III parvalbumin which are not involved in the CD or EF calcium-binding sites. Of these, only three, namely, Glu-60, Asp-61, and Asp-100, are within 20 Å of either the CD or EF sites. Of these three, only Asp-61, which is 7.3 Å from the CD site and 13.5 Å from the EF site, appears to be in a favorable position for participation in a third binding site. Inspection of the three-dimensional structure with the aid of stereoscopic projections (Feldmann, 1976) reveals that there are three carboxylate oxygen atoms, one each from Asp-53, Asp-59, and Asp-62, all within 6 Å of the oxygen atoms of Asp-61, which are favorably positioned for the coordination of a Ln(III) ion. Since Asp-53, -59, and -62 are coordinated to the metal ion in the CD site, the postulated third site would involve carboxylate bridges between ions in the third site and the CD site. Precedence for such a bridged structure may be found in calcium sites 1 and 2 of thermolysin (Matthews et al., 1974; Matthews & Weaver, 1974). A stereoview of the region encompassing the CD site and the postulated third site is shown in Figure 6. The above-suggested third site is entirely consistent with about three water molecules coordinated to a Ln(III) ion occupying this position.

Mechanism of Quenching by an Ion Occupying the Third Site. While our experiments do not allow us to establish unambiguously a mechanism of quenching, certain possibilities can be eliminated and possible mechanisms, consistent with our observations, can be postulated. Since in H_2O solution the quenching occurs for both Tb(III) and Eu(III) regardless of whether sensitization occurs through the protein or by direct excitation of metal ion levels, a conformational change affecting the phenylalanine to Ln(III) energy transfer upon binding to the third site can not alone explain the results (Figure 2). On the other hand, since the postulated third site is intimately connected to the CD site by carboxylate bridges and less directly via the CD coordination sphere through the hydrogen bond between the NH of Ile-58 of the CD site and the peptide carbonyl of Ile-97 involved in the EF site, changes in these coordination sites might be expected upon occupation of the third site. Such changes might decrease the quantum yields of Ln(III) emission in the CD and EF sites by changing the ligand field or by permitting the coordination of an additional water molecule at these sites. The decreased quenching in D_2O solution for sensitization through the protein (Figure 2A,B) is consistent with the latter postulate. An additional quenching mechanism is available for an ion in the CD site, namely, direct electronic energy transfer through bridging carboxylate ligands to its counterpart in the third site which, owing to the possession of more coordinated water molecules, is expected to have a lower quantum yield. The titration results for direct excitation of Ln(III) ion levels in D_2O (Figure 2A,B), which do not show the quenching observed for the other systems, can be accounted for as follows. The third site, which is not significantly sensitized via protein chromophores, makes a significant contribution to the total luminescence when direct excitation is used. The underlying quenching is thus masked by this additional contribution from direct excitation of the third site which has a reasonably high quantum yield in D_2O solution. The more usual result is obtained in H_2O solution owing to the much lower quantum yield of the third site Ln(III) ion. The preceding ideas regarding the quenching mechanism and the constitution of the third site should be considered as tentative suggestions only, but they are, at least, in accord with the experimental facts now known. In any case, complications from the occupation of the third site can be eliminated by carrying out experiments at low pH. This was done in our study of energy transfer between Ln(III) ions occupying the CD and EF sites which is discussed in the next section.

Analysis of Inter-Ln(III)-Ion Energy Transfer. As a working hypothesis it will be assumed that energy transfer from a donor metal in one of the primary sites (CD or EF) to an acceptor ion in the other primary site occurs by a Förster-type dipole–dipole mechanism. It will also be assumed that the

actual distance between these sites is reasonably close to the 11.8 Å found in the X-ray structure of the native protein. Since we are unable selectively to substitute one of the sites with one Ln(III) ion and the other site with another Ln(III) ion, transfer of energy from the CD to EF sites and vice versa cannot be distinguished and are treated as one and the same. The similar lifetimes and nearly coincident excitation peaks for Eu(III) ions occupying these two sites suggest similar emission properties for ions in the two environments. The experimentally determined energy-transfer efficiencies determined from the reciprocal lifetime results for various donor–acceptor pairs (Table I) range from 0 for nonacceptor ions to 54% for the Tb(III)–Nd(III) couple. Our approach is to estimate the interionic distances from our energy-transfer results and to compare these with the X-ray result. R_0 values were estimated as follows. κ^2, the orientation factor, is taken as $^2/_3$, the result when both the donor emission and acceptor absorption are isotropic. This is likely to be a good assumption since both emission and absorption involve transitions between the very slightly split components of highly degenerate free ion levels. For the refractive index, n, of the intervening medium a value of 1.35 has been chosen. This is intermediate between that of water (1.33) and that of organic molecules containing only first row atoms (1.39). In the absence of absorption spectral data for protein-bound Tb(III) and Eu(III), only estimates of the quantum yields, ϕ, can be obtained. The excited-state lifetime ratios, τ_{H_2O}/τ_{D_2O}, provide upper limit values of 0.54 and 0.36 for Tb(III) and Eu(III), respectively. The actual values are undoubtedly somewhat lower, and values decreased by 22% from these maxima are chosen for use in the present calculations. Since the distance estimate involves taking the sixth root of R_0^6, minor uncertainties in these parameters are not critical. The choice of a parameter differing by as much as a factor of 2 will cause only a 12% change in the distance estimate (±1.5 Å in the present case).

Although in principle it can be experimentally determined, the spectral overlap integral, J, is the most difficult component of R_0 to estimate. The extremely low molar extinction coefficients generally preclude the accurate measurement of absorption spectra of Ln(III) ions bound to proteins. Recourse must be taken to model complexes and the spectral overlap integrals computed by using protein emission and model complex absorption using data such as illustrated in Figure 5. Unfortunately, for a given donor–acceptor pair the J values calculated by using a variety of model complexes span a considerable range (Horrocks et al., 1980). Further discussion concerning the evaluation of these overlap integrals can be found in the following paper (Snyder et al., 1981). In the present analysis we use the J values calculated by using $[Ln^{III}(DPA)_3]^{3-}$ complexes, which lie at the upper end of the range found for the various models. It is gratifying that those systems for which J is 0 or negligibly small exhibit no measurable energy transfer. For instance, for Eu(III) as a donor and Ho(III) and Er(III) as acceptors the J values are calculated to be 0.68×10^{-18} and 0.74×10^{-18} cm^6 mol^{-1}, respectively, corresponding to R_0 values of 5.7 and 5.8 Å, respectively, in H_2O solution. No measurable energy transfer is observed in either case. While the same acceptor ions with Tb(III) as a donor have much larger J values which lead to R_0 values in the 8–10-Å range and readily measurable energy-transfer efficiencies (Table I). The R_0 values for all the systems which exhibited measurable energy transfer are set out in Table I. The values of the donor–acceptor separation, r, calculated from the measured efficiencies and the R_0 values are shown in the final two columns of Table I.

In the case of Eu(III) as a donor, the two r values estimated average 12.1 Å, which is 0.3 Å greater than the CD–EF Ca(II) ion separation in the crystal structure. For Tb(III) as a donor, the estimated r values average 9.4 Å, which is 2.4 Å shorter than the X-ray distance. The answer to the question of whether use of Tb(III) as a donor will in general give results which are systematically shorter than those obtained by using Eu(III) must await further work. In any case, our results suggest that inter-Ln(III)-ion energy transfer measurements will be useful in measuring distances between Ca(II) binding sites in proteins with an uncertainty of 10–15% for distances not too dissimilar from the corresponding R_0 values.

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Lanthanide Ion Luminescence Probes. Characterization of Metal Ion Binding Sites and Intermetal Energy Transfer Distance Measurements in Calcium-Binding Proteins. 2. Thermolysin†

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ABSTRACT: Eu(III) laser excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition reveals spectral features characteristic of the occupation of Eu(III) ions in the S(1), S(3), and S(4) Ca(II)-binding sites of thermolysin. Various hybrid Eu(III) thermolysin species were prepared in which the Eu(III) ion resides in the S(1) or S(3) and S(4) or all three Eu(III)-binding sites of the metalloprotein. The Eu(III) ion in the S(1) site coordinates to one H_2O molecule while the other two sites coordinate to about three to four H_2O molecules. Thermolysin hybrids in which the donor Ln(III) ion occupies S(1) and acceptor Ln(III) ions occupy sites S(3) and S(4) were prepared. Nonradiative energy transfer between Eu(III) and Tb(III) acting as luminescent donors and various other Ln(III) ions serving as acceptors was observed by monitoring the excited-state lifetimes of the donor ions using a pulsed dye laser apparatus. Virtually all energy transfer occurs between sites S(1) and S(4) while <1% of the donor's energy is transferred from S(1) to S(3). With the assumption of a Förster-type dipole–dipole mechanism, the experimental inter-binding-site distance estimates between sites S(1) and S(4) are in good agreement with the distance (11.7 Å) obtained by X-ray crystallography. R_0 values (critical distances for 50% energy transfer) in H_2O solution range from 9.3 Å for the Tb(III)–Ho(III) donor–acceptor pair down to 5.3 Å for the Eu(III)–Ho(III) pair.

In this, as in the preceding paper (Rhee et al., 1981), we seek to characterize the calcium-binding sites of a structurally well-characterized protein by using luminescence spectroscopic techniques developed in this laboratory (Horrocks & Sudnick, 1979a,b). Also we wish to establish the utility of the measurement of energy transfer between trivalent lanthanide ions, Ln(III), in determining distances between metal ion binding sites. Stryer (1978) and Steinberg (1971) have reviewed other types of energy-transfer distance measurement. Thermolysin (EC 3.4.24.4), a thermostable, calcium-binding zinc metalloendoprotease from *Bacillus thermoproteolyticus*, the subject of the present paper, is ideal for our purposes. Its amino acid sequence (Titani et al., 1972) and three-dimensional X-ray structure to a resolution of 2.3 Å (Matthews et al., 1972, 1974) have been determined. Moreover, a detailed study of the binding of Ln(III) ions at the calcium-binding sites, S(1), S(3), and S(4), has been carried out by using crystallographic techniques (Matthews & Weaver, 1974). The structural and functional roles of metal ions in thermolysin have recently been reviewed (Roche & Voordouw, 1978).

An earlier study by one of us (Horrocks et al., 1975) demonstrated by using ordinary fluorometry the occurrence of energy transfer between a luminescing Tb(III) occupying calcium site S(1) and an absorbing Co(II) substituted for Zn(II) at the active site of thermolysin. With the assumption of a Förster-type dipole–dipole mechanism (Förster, 1948, 1965), an estimate of the distance between Tb(III) and Co(II) of 13.7 Å was made, which was in good agreement with the distance between the Ca(II) ion in site S(1) and the active site Zn(II) ion found in the X-ray structure (Matthews et al., 1974). In the present study, energy transfer between emitting donor and absorbing acceptor Ln(III) ions occupying different calcium-binding sites is monitored via the reciprocal excited-state lifetimes of the luminescent donor ions using a pulsed dye laser excitation technique. This study is aided by the fact that it is possible to substitute selectively one type of Ln(III) ion, say Eu(III) in site S(1), and another type, say Nd(III), in sites S(3) and S(4).

Some uncertainty exists in the literature regarding the mechanism of inter-Ln(III)-ion energy transfer, e.g., whether it occurs by a dipole–dipole, dipole–quadrupole, or quadrupole–quadrupole mechanism (Reisfeld, 1975; Reisfeld, 1976; Nakazawa & Shionoya, 1967; Grant, 1971; Fong & Diestler, 1972). This research, which in effect uses a metalloprotein model to address this problem in photophysics, settles this controversy in favor of a dipole–dipole mechanism. Since our techniques are applicable to all states of matter, direct solution-state–solid-state comparisons can be made. A preliminary report of some of our findings has appeared elsewhere (Horrocks et al., 1980).

Materials and Methods

Materials. Thermolysin and deuterium oxide (99.7%) were purchased from Sigma Chemical Co. Ln(III) salts were obtained from either Research Organic/Inorganic or Alfa-

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