as anodes and cathodes. It appears that this wireless photoelectrolysis could be the simplest means of large scale solar energy harnessing and conversion.

I wish to express my deep gratitude to my collaborators as well as to my Italian colleagues, Professor E. Pelizzetti and Dr. M. Visca, whose inspired and enthusiastic effort has made possible the success of this work. Financial assistance of the Swiss National Foundation, Ciba Geigy, and Engelhard Industries is also gratefully acknowledged.

Lanthanide Ion Luminescence Probes of the Structure of Biological Macromolecules

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Approximately a third of all proteins require metal ions for their activity or contain bound metal ions in their native states.[1-3] Some of these metal ions, e.g., Cu, Fe, Mo, exhibit useful spectroscopic or magnetic probe properties, while others, e.g., Zn, Mg, Ca, do not. When the native metal ion is devoid of useful properties, it is often possible to substitute an ion with useful characteristics. Some examples are the replacement of Zn(II) by Co(II)[4,5] or Ca(II) by the trivalent lanthanide ions.[6-10] This latter substitution is the principal concern of this Account.

In any study in which a biomolecule is modified, it is important to establish what, if any, changes occur upon substitution. The ideal probe will leave the macromolecule virtually indistinguishable from the native species except for the spectroscopic "handle". For enzymes, the retention of at least some degree of biological activity is evidence that the native conformation has not been drastically altered. Lanthanide ions (symbolically Ln(III)) have been reported to activate the following systems to their biological function in the absence of Ca(II): aequorin,[11] concanavalin A,[12] trypsinogen,[13] phospholipase A_2,[14] α-amylase,[15] galactosyltransferase,[16] and prothrombin (activation by activated factor X).[17] In the cases of staphylococcal nuclease,[18] phosphoglycerate kinase,[19] and calcium ATPase[20] and in the activation of bovine factor X by the coagulant protein of Russell's viper venom,[21] Ln(III) ions act as inhibitors of the Ca(II) or Mg(II) ion functions. Ln(III) ion substitution has no effect on the enzymatic activity of thermolysin[22] and elastase.[23] In addition, Ln(III) ions have been shown by X-ray crystallographic techniques to substitute isomorphously for Ca(II) in thermolysin[24] and parvalbumin.[25,26] It can be argued that replacement of a metal by one of similar size represents a smaller perturbation of a macromolecule than the chemical modification of a polypeptide side chain using, say, a nitroxide spin-label.

The trivalent ions of the 14 stable elements from La(III) through Lu(III) have ionic radii ranging from slightly greater to slightly smaller than that of Ca(II).[27]

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Also, in common with Ca(II), Ln(III) ions exhibit a preference for oxygen donor ligands, have coordination numbers generally greater than six, and show little stereochemical preference in their coordination chemistry. The additional charge on Ln(III) ions is not a deterrent to their replacing Ca(II). Macromolecules with their charged groups and counterions are able to adjust to the gain of a single positive charge without significant structural consequences. This is evidenced by X-ray studies on Ln(III)-substituted calcium-binding proteins.[24-26] The only significant chemical differences are slower ligand exchange rates[28] and greater binding constants[29] for the Ln(III) ions. This latter quality is of considerable aid in effecting substitution for Ca(II) ions. The decreased lability of Ln(III) ions has important consequences when Ln(III) ions occupy metal substrate-binding sites in calcium-requiring enzymes.

With the exceptions of La(III) ($4f^0$) and Lu(III) ($4f^{14}$) all of the Ln(III) ions are open-shell, paramagnetic species. The terms of the various $4f^n$ configurations give rise, in most cases, to low-lying excited states which cause visible absorption. Individual terms are split into various J manifolds by the sizable spin–orbit coupling interaction. The J levels are in turn split by the ligand field into as many as $2J + 1$ closely spaced components.[30] Certain excited states of many of the Ln(III) ions are able to relax by the emission of photons. Indeed the Ln(III) ions are distinguished among metallic cations in their ability to luminesce in solution at room temperature. Since luminescence can be detected with high sensitivity, heavy use has been made of protein fluorescence and organic fluorescent probes in biochemical research.[31] This Account relates our efforts to develop and exploit metal ion luminescence as a probe of biological macromolecules.

Laser-Induced Lanthanide Ion Luminescence

Electronic transitions within a $4f^n$ configuration are Laporte forbidden, causing Ln(III) ion luminescence to be feeble compared with the fluorescence of organic fluorophores, a drawback for experiments carried out on dilute solutions of biological macromolecules. We use an intense laser beam as an excitation source to overcome this difficulty, allowing us to detect Ln(III) ion luminescence from solutions as dilute as 1 μM and below.

Eu(III) and Tb(III) are the most strongly emitting members of the Ln(III) ion series, and our studies have been devoted exclusively to them. Energy level diagrams for Eu(III) and Tb(III) are shown in Figure 1. The $^5D_0$ and $^5D_4$ emissive levels of Eu(III) and Tb(III) can be excited by 579- and 488-nm light, respectively. The use of visible light for the excitation eliminates problems of protein photosensitivity. The excited-state lifetimes of these ions are environmentally sensitive and lie in the conveniently long 100–3000-μs range. While ligand field splittings of f-electron levels are much smaller than for the d-electronic levels of transition-metal complexes, the absorption bands are generally much narrower, and small splittings can be readily

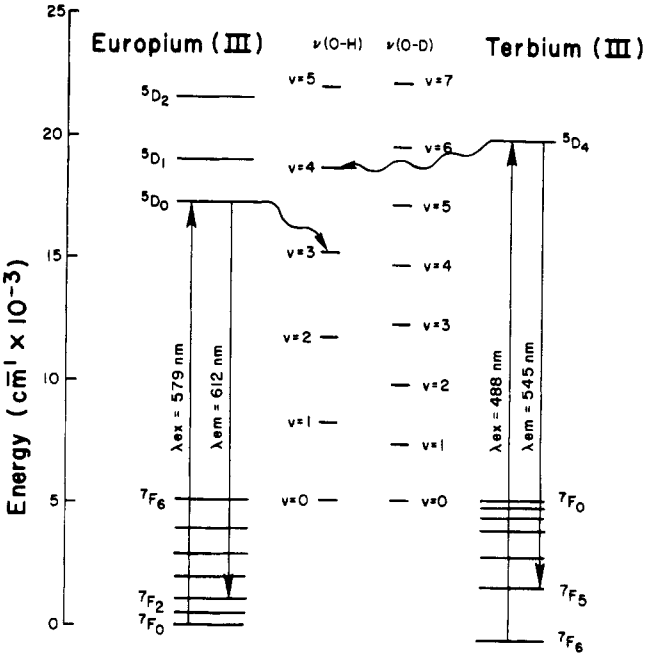

Figure 1. Electronic energy levels for europium(III) and terbium(III). The two upward-pointing arrows show the transitions which occur upon laser excitation at the wavelengths indicated. The two downward-pointing arrows label the most intense emissive transitions of the two ions. Radiationless energy transfer competes with the radiative processes through coupling of the emissive states to the O–H vibrational overtones of coordinated H_2O molecules. (The energy level diagram of Tb(III) has been displaced slightly to position the highest electronic acceptor state of the ground ^7F term to coincide with the zero-point energies of the vibrational overtone ladders.)

detected. Individual emission bands can be examined under high resolution to yield information about the splitting of ground and excited states. Excitation spectroscopy using a tunable laser also yields information of this type.

Instrumentation

Space limitations permit only a very brief description of the experimental setup currently in use, although most of our published results were obtained on a somewhat less sensitive apparatus. Our experiment is very simple. The pulsed ($\sim$10 s^{-1}) output of a nitrogen laser pumped dye laser is focused onto the sample held in a quartz cuvette or other container (sample volumes as small as 30 μL have been used successfully). Luminescence is sampled at 90° with a lens to focus the light onto the slits of a 0.2-m double monochrometer set to accept the most intense emission band (Figure 1). This monochrometer discriminates efficiently against scattered laser light. The signal is detected by using a photomultiplier tube equipped with a thermoelectrically cooled housing and employing photon-counting electronics. Luminescence decays from successive laser pulses are recorded on a multichannel device and accumulated until a satisfactory signal to noise ratio is achieved. The luminescence decay data are read out on an x-y recorder or transferred to magnetic tape for later analysis by computer.

Excitation spectra, wherein the dye laser is scanned continuously through an electronic absorption band while the luminescence emission intensity is monitored, are obtained on the same apparatus with only slight modifications. Appropriately gated photon emission

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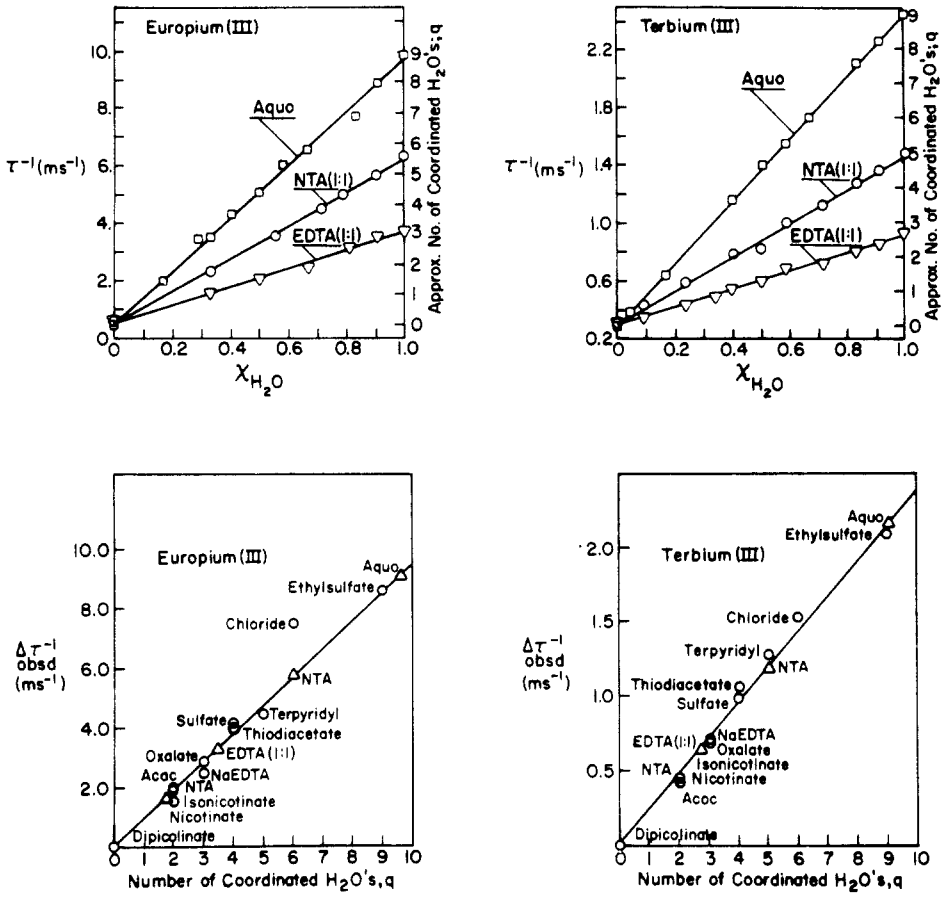

Figure 2. (Top) Plots of the observed reciprocal luminescence lifetimes, τ^{-1}_{obsd}, vs. the mole fraction of H_2O, χ_{H_2O}, in D_2O–H_2O mixtures of Eu(III) and Tb(III) solutions, respectively. The effect of the chelating ligands NTA and EDTA is to displace water molecules from the first coordination spheres of the aquametal ions to yield the approximate values indicated along the right-hand ordinate of each figure. (Bottom) Plots of the difference in the observed reciprocal luminescence lifetimes, $\Delta\tau^{-1}_{obsd}$ ($=\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O}$) for well-characterized crystalline hydrates of Eu(III) and Tb(III) (open circles). These differences are plotted vs. the crystallographically determined numbers of water molecules in the first coordination spheres, q. The open triangles are the $\Delta\tau^{-1}_{obsd}$ values for each of the three solution complexes (top). These differences have been placed on the least-squares line for each of the crystalline hydrate data sets.

events following each laser pulse are counted or summed in successive channels of the signal averager as the excitation wavelength is continuously scanned. The resulting trace tracks the absorption band of the species emitting photons. This spectroscopic technique represents perhaps the most sensitive spectroscopic means (not involving radioactivity) of directly detecting bound metal ions. For instance, the excitation spectrum of the $^5D_0 \leftarrow {}^7F_0$ transition of Eu(III) bound to thermolysin at a concentration of 0.18 μM was recorded in about 10 min (Figure 5D).

Luminescence Decay Lifetimes Provide a Direct Measure of the Number of Metal-Coordinated Water Molecules

Several processes contribute to the deexcitation of an excited-state ion. An exponential decrease of luminescence intensity following excitation is usually observed. The experimental reciprocal excited-state lifetime (exponential decay constant), τ^{-1}_{obsd}, is comprised of several terms (eq 1), where τ^{-1}_{nat} is the natural

$$\tau^{-1}_{obsd} = \tau^{-1}_{nat} + \tau^{-1}_{nonrad} + \tau^{-1}_{OH} \qquad (1)$$

rate constant for the emission of photons, τ^{-1}_{nonrad} represents the rate constant for nonradiative deexcitation which does not involve OH oscillators, and τ^{-1}_{OH} is the rate constant for nonradiative energy transfer to the OH vibrational manifold of OH oscillators in the first coordination sphere (e.g., coordinated water molecules)). τ^{-1}_{OH} is quite significant. For instance, for the Tb^{3+}(aq) ion $\tau^{-1}_{nat} = 0.11$, $\tau^{-1}_{nonrad} = 0.19$, and $\tau^{-1}_{OH} = 2.15$ ms^{-1} while for Eu^{3+}(aq) the corresponding values are $\tau^{-1}_{nat} = 0.19$, $\tau^{-1}_{nonrad} = 0.25$, and $\tau^{-1}_{OH} = 9.5$ ms^{-1}.[32-34] While τ^{-1}_{nat} and τ^{-1}_{nonrad} are comparable for the two ions, τ^{-1}_{OH} is considerably greater for Eu^{3+}(aq). Figure 1 indicates schematically the nonradiative transfer of energy from the excited states of Tb(III) and Eu(III) to the OH vibrational manifold. The efficiency of this transfer increases as the magnitude of the energy gap between the emissive state and the highest level of the ground manifold decreases.[33,34] This gap is about 14 800 cm^{-1} for Tb(III) and 12 200 cm^{-1} for Eu(III), accounting for the markedly greater efficiency in the latter case. There is a major isotope effect on this energy transfer. Replacement of OH oscillators by the OD variety causes the vibronic deexcitation pathway to become exceedingly inefficient.[34-41] This fact enables one to determine

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the number of OH oscillators in the first coordination sphere by carrying out experiments independently on H_2O and D_2O solutions. In D_2O solution τ^{-1}_{OH} vanishes and τ^{-1}_{obsd} equals $\tau^{-1}_{nat} + \tau^{-1}_{nonrad}$, where the latter term includes any small deexcitation via OD oscillators. τ^{-1}_{obsd} varies linearly with the mole fraction of H_2O, χ_{H_2O}, in H_2O–D_2O mixtures (Figure 2).[42] This allows the extrapolation of τ^{-1}_{obsd} values to $\chi_{H_2O} = 0$ (pure D_2O) to obtain $\tau^{-1}_{nat} + \tau^{-1}_{nonrad}$. These values along with a measurement in pure H_2O yield τ^{-1}_{OH}, which is proportional to the number of OH oscillators (or H_2O molecules) in the first coordination sphere. There is a dramatic reduction of τ^{-1}_{OH} (and of τ^{-1}_{obsd}) upon the exclusion of water molecules from the first coordination sphere by multidentate ligands such as ethylenediaminetetraacetate (EDTA) or nitrilotriacetate (NTA) (Figure 2 (top)). Approximate scales indicating the number of water molecules remaining in the first coordination spheres of Eu(III) and Tb(III) are shown in this figure.

Measurements of this type are not limited to the solution state, and experiments on a series of structurally well characterized crystalline solids further substantiate the method.[43] Figure 2 (bottom) shows plots of the difference, $\Delta\tau^{-1} = \tau^{-1}_{H_2O} - \tau^{-1}_{D_2O}$, in the τ^{-1}_{obsd} values measured on crystalline Ln(III) complexes grown separately from H_2O and D_2O solutions vs. the number of water molecules in the first coordination sphere (obtained from X-ray crystallography). A good linear correlation between the $\Delta\tau^{-1}$ values and the number of coordinated water molecules is evident. Equation 2 describes the best least-squares lines

$$q_{Ln} = A_{Ln}(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O}) \qquad (2)$$

through the solid-state data points. In eq 2, q represents the number of water molecules coordinated to the particular Ln(III) ion ($A_{Eu} = 1.05$, $A_{Tb} = 4.2$; τ^{-1} values in ms^{-1}). The estimated uncertainty in q is approximately ±0.5 water molecules. Equation 2 can be used to interpret solution state results on H_2O and D_2O solutions of various Ln(III) ion systems including protein-bound species. The determination of q provides inferential evidence regarding the number of groups on the protein which coordinate to the metal ion. Since the $(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O})$ values for Eu(III) are more than four times as great as for Tb(III), measurements on the former ion provide a particularly sensitive means of measuring the number of coordinated water molecules, especially when used in combination with the spectroscopic method capable of distinguishing individual, distinct metal-binding sites described below.

Eu(III) Excitation Spectroscopy. Characterization of Individual Binding Sites[44]

The Eu(III) ion is unique in that both the ground state ($^7F_0$) and excited emissive state ($^5D_0$) are nondegenerate (see Figure 1). Since neither of these levels can be split by a ligand field, the absorption band corresponding to a transition between these levels must consist of a single, unsplit line for a given Eu(III) ion environment. Different ionic environments can, in principle, yield transitions at slightly different frequencies. Owing to the extremely low molar extinction coefficient ($\sim 10^{-2}$ M^{-1} cm^{-1}) for the highly forbidden $^5D_0 \leftarrow {}^7F_0$ transition, study of this band by ordinary absorption spectroscopy on dilute solutions is not feasible. However, since the $^5D_0$ level is emissive, this transition can be studied via excitation spectroscopy by monitoring emitted photons ($^5D_0 \rightarrow {}^7F_2$, 612 nm) while a tunable laser is continuously scanned through the $^5D_0 \leftarrow {}^7F_0$ transition region (578–580 nm). Even in solution at room temperature excitation spectral peaks are quite sharp with line widths at half-maximum as small as ~ 8 cm^{-1}, allowing the resolution of spectra which consist of several nearly coincident transitions.

The titration of the Eu(III) aqua ion with the tridentate ligand, dipicolinate (DPA), provides an exam-

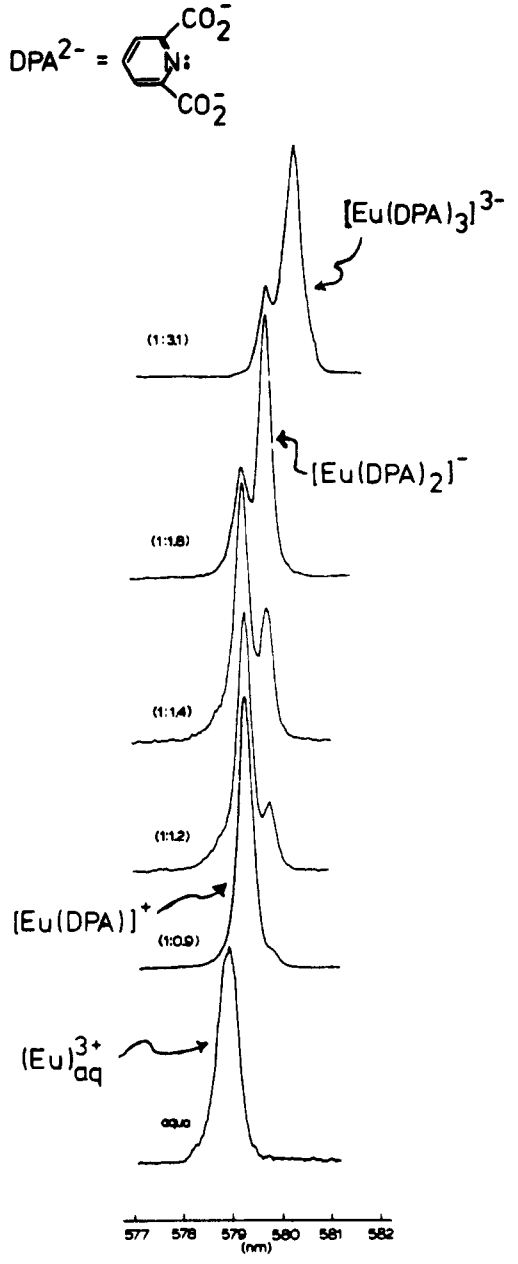

Figure 3. Excitation spectral profiles of the $^7F_0 \rightarrow {}^5D_0$ transition obtained during the course of the titration of an aqueous $EuCl_3$ solution with the sodium salt of the dipicolinate anion (DPA). Eu(III) to ligand ratios are indicated to the left of each trace.

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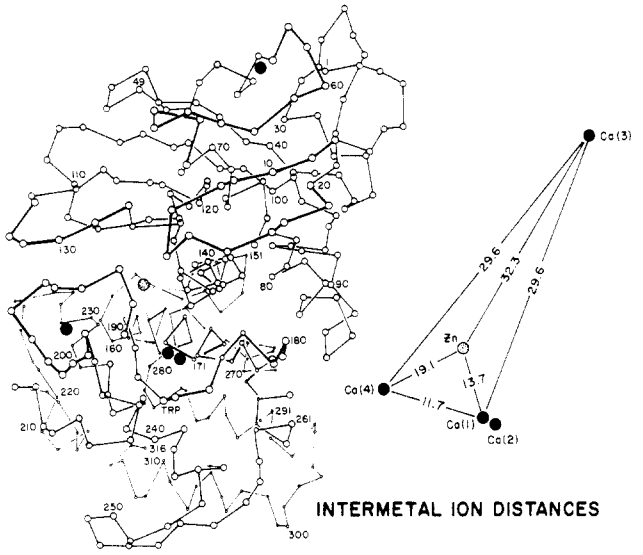

Figure 4. X-ray structure of thermolysin (adapted from ref 48). The open circles represent the position of the α-carbon atoms in the polypeptide chain. The heavy dark circles represent the positions of the calcium ions in the native protein. The stippled circle represents the zinc ion at the catalytically active site. The schematic diagram at the right depicts the inter metal ion distances.

ple[44] of how this method can be applied to the detection and characterization of individual Eu(III) ion environments. Figure 3 shows the excitation spectral profiles as successive amounts of DPA are added to a solution of europium chloride. Individual narrow peaks characteristic of $Eu^{3+}(aq)$, $[Eu(DPA)]^+$, $[Eu(DPA)_2]^-$, and $[Eu(DPA)_3]^{3-}$ are apparent at the following respective frequencies: 17 272, 17 263, 17 248, and 17 231 cm^{-1}. In addition to providing a relatively high-resolution means of detecting individual coordination complexes in solution, the excitation peaks are, in effect, labeled by their individual reciprocal lifetimes, τ^{-1} (possible in this case because the rates of chemical interconversion between complexes are much less than the τ^{-1} values). The four species present at one time or another during the titration, listed in the above order, have the following τ^{-1}_{obsd} values: 9.62, 5.92, 3.29, and 0.61 ms^{-1}. These in combination with the results of a parallel titration carried out in D_2O solution yield the following estimates for the number of Eu(III)-coordinated water molecules in the respective species: 9.7, 5.9, 3.1, and 0.3. These numbers are in good accord with the expectation that Eu(III) will adopt a total coordination number of 9 or 10 and that each tridentate DPA ligand will displace three water molecules. Eu(III) excitation spectroscopy has also found utility in characterizing Eu(III) ion environments in crystalline matrices and amorphous solids.[45,46]

Our initial experiments on macromolecular systems have been carried out on calcium-binding proteins for which Ln(III) ion binding has been studied by X-ray crystallography. One such protein is thermolysin,[47] a thermostable endoproteinase which binds a Zn(II) ion at the active site and four structural Ca(II) ions[48,49]

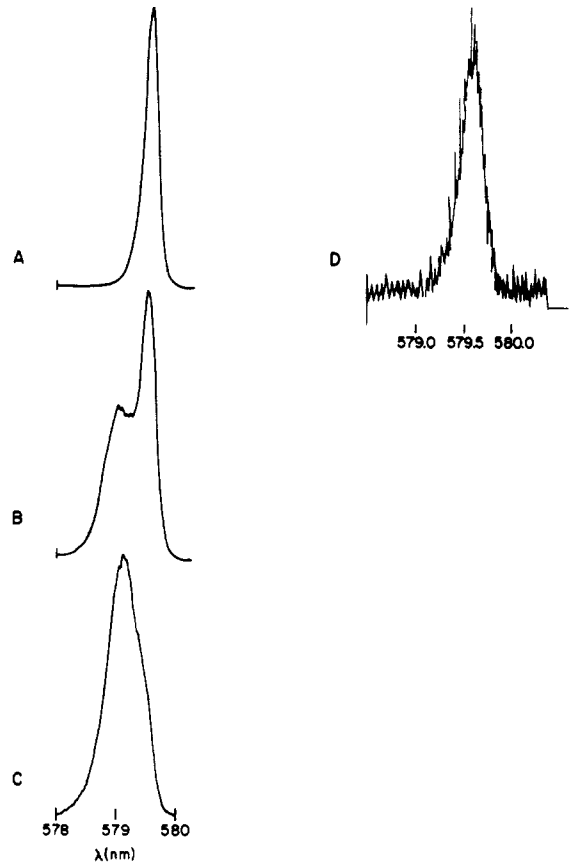

Figure 5. Excitation ($^7F_0 \rightarrow {}^5D_0$) profiles of various Ln(III)-substituted thermolysin derivatives in solution (~0.4 mM). (A) Eu[1]Ca[3,4]TL; (B) Eu[1,3,4]TL; (C) Dy[1]Eu[3,4]TL; (D, 0.18 μM) Eu[1]Ca[3,4]TL.

(Figure 4). The Zn(II) ions, required for activity, can be replaced by other divalent ions such as Co(II), in which case the catalytic activity is enhanced.[22,50] Either one or three Ca(II) ions can be replaced by Ln(III) ions with no effect on activity.[22,51] It is known[24,52] that if 1 equiv of a Ln(III) ion is added to thermolysin, this ion will bind strongly at calcium site S(1) with the concomitant expulsion of the Ca(II) ions at sites S(1) and S(2), while Ca(II) ions remain at sites S(3) and S(4). This metallo derivative is designated Ln[1]Ca[3,4]TL, using an obvious notation. On the other hand, if crystals of thermolysin are soaked in Ln(III) ion containing solutions,[24,52] Ln(III) ion substitution at sites S(1), S(3), and S(4) is achieved (Ln[1,3,4]TL). Significantly, it is possible to make selective substitutions in the following manner:[51] Ln[1]Ca[3,4]TL is prepared as described above and crystallized; these crystals are then soaked in a buffer containing a different lanthanide ion, Ln′(III), resulting in the formation of the hybrid species Ln[1]Ln′[3,4]TL. Owing to the virtually exchange-inert nature of Ln(III) ion binding to site S(1), minimal replacement of the ion at this site results.

The potential of Eu(III) excitation spectroscopy[44] for the characterization of individual metal ion binding

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sites is illustrated in Figure 5. The uppermost trace (A) shows the spectrum of Eu[1]Ca[3,4]TL, while the middle trace (B) shows that of Eu[1,3,4]TL. Additional features at higher energy due to occupation of sites S(3) and S(4) by Eu(III) ions are clearly evident. The lowermost trace (C) depicts the spectrum of Dy[1]Eu[3,4]TL. Since Dy(III) is silent in this experiment, only the features due to Eu(III) at sites S(3) and S(4) are evident, save perhaps for a trace contaminant with Eu(III) at site S(1).

The various Eu(III) coordination environments can be further characterized by their excited-state reciprocal lifetimes. The τ^{-1} values for excitation into the sharp signal at 579.5 nm of Eu[1]Ca[3,4]TL are $\tau^{-1}_{H_2O} = 1.78$ ms^{-1} and $\tau^{-1}_{D_2O} = 0.61$ ms^{-1}. These findings lead (eq 2, Ln = Eu) to the conclusion that the number of water molecules coordinated to Eu(III) at this site, q, is 1.2 ± 0.5. Excitation into the feature at 579.1 nm of Eu[1,3,4]TL or Dy[1]Eu[3,4]TL yielded luminescence decay curves that could be resolved into two separate exponential components leading to q values of 4.0 and 3.1. Both results are in agreement with the X-ray finding of one water molecule bound at S(1) and three at both S(3) and S(4), with the OH oscillator of a coordinated threonine contributing in the latter case as well.

Other proteins characterized in our laboratory by this excitation spectroscopic technique include parvalbumin,[53,54] troponin C,[55] and calmodulin.[56] The ability of this technique to characterize individual Eu(III) ion binding sites at submicromolar concentrations by both $^5D_0 \leftarrow {}^7F_0$ transition energies and excited-state lifetimes places it among the more sensitive methods for the investigation of metal-binding macromolecules.

Inter Metal Ion Energy Transfer Distance Measurements

When a luminescent energy donor, D, and an absorbing energy acceptor, A, reside within the same molecule (e.g., a protein) and there is significant overlap between the emission spectrum of D and the absorption spectrum of A, energy transfer of a nonradiative kind can take place between the two moieties. For D–A separations far less than the wavelength of the light involved, ordinary emission followed by absorption of electromagnetic radiation is not possible. Förster[57,58] showed that the efficiency of dipole–dipole energy transfer, E, is inversely proportional to the sixth power of the D–A separation, r. Thus, if it is possible to monitor this energy transfer experimentally, a measure of r can be obtained. Förster-type nonradiative energy transfer affects both the observed luminescence intensity, I, and the excited-state lifetime, τ, of the donor. The efficiency, E, is given by eq 3, where the subscript zero denotes the value of the quantity in the absence of energy transfer. The measured efficiency is related to the actual D–A separation, r, by eq 4, where the

$$E = 1 - \frac{I}{I_0} = 1 - \frac{\tau}{\tau_0} \tag{3}$$

$$E = \left[1 + \left(\frac{r}{R_0}\right)^6\right]^{-1} \tag{4}$$

critical distance for 50% energy transfer, R_0, is given by eq 5. κ^2 is the orientation factor, ϕ is the quantum

$$R_0^6 = 8.78 \times 10^{-25}\, \kappa^2 \phi n^{-4} J \tag{5}$$

yield of D in the absence of A, and n is the refractive index of the intervening medium. J is the spectral overlap integral defined by eq 6, where $F(\nu)$ is the luminescence intensity of D, $\epsilon(\nu)$ is the molar extinction coefficient of A in units of M^{-1} cm^{-1}, and ν is the frequency in cm^{-1}.

$$J = \frac{\int F(\nu)\epsilon(\nu)\nu^{-4}\, d\nu}{\int F(\nu)\, d\nu} \tag{6}$$

Energy transfer measurements have been used with considerable success to measure distances between organic fluorophores and organic absorbing chromophores when these are attached to proteins.[59,60] The first successful observations[22,61] of energy transfer between metal ions bound to a protein were made in 1975, although this phenomenon had been searched for earlier.[62] Energy transfer between a Tb(III) ion occupying site S(1) of thermolysin and a Co(II) ion bound at the Zn(II) site occurs[22] with an efficiency of about 0.90. The spectral overlap integral, J, calculated from the emission spectrum of Tb(III) and the absorption spectrum of bound-Co(II) (Figure 6A) of 5.96×10^{-16} cm^6 mol^{-1} along with the estimates of the other parameters in eq 5 leads to an R_0 value of 19.6 Å. These results predict a Tb(III)–Co(II) distance of 13.7 Å, in excellent agreement with the X-ray distance[49] from site S(1) to the Zn(II) site. In the evaluation of R_0 a κ^2 value of $^2/_3$, its isotropic limit, was taken. This is probably a good approximation for metal ions owing to the degeneracy or near-degeneracies in their energy levels which cause the electronic transitions not to be polarized exclusively in a particular direction.

Other recent examples of inter metal ion energy transfer in proteins involve Tb(III) to Fe(III) transfer in transferin[63] ($R_0 = 27.1$ Å, $r = 25 \pm 2$ Å) and Eu(III) to Co(II) transfer in galactosyltransferase[16] ($R_0 = 20$ Å, $r = 18 \pm 3$ Å). In neither case are confirmatory crystallographic studies available.

Inter Ln(III) Ion Energy Transfer. Calcium-binding proteins are ubiquitous and important.[64-66] Many have multiple Ca(II) coordination sites. In order to explore distance relationships in this class of protein, we have set out to measure energy transfer between

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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 the Experimentally Estimated Distances, r, between Sites S(1) and S(4) of Thermolysin for Various Donor-Acceptor Ln(III) Ion Pairs

donor	acceptor	state	τ^{-1}, ms^{-1}	E	$J \times 10^{18}$ cm^6 mol^{-1} [a]	R_0, Å [b]	r, Å [c]
Eu(III)	Pr(III)	solution	1.98	0.101	6.50	8.20	11.8
Eu(III)	Pr(III)	solid	2.07	0.111	6.50	8.20	11.6
Eu(III)	Nd(III)	solution	2.12	0.160	8.26	8.53	11.2
Eu(III)	Nd(III)	solid	2.21	0.167	8.26	8.53	11.2
Tb(III)	Pr(III)	solution	0.78	0.115	2.66	7.77	10.9
Tb(III)	Pr(III)	solid	0.84	0.107	2.66	7.77	11.1
Tb(III)	Nd(III)	solution	1.62	0.587	6.95	9.12	8.6
Tb(III)	Nd(III)	solid	1.67	0.551	6.95	9.12	8.8
Tb(III)	Ho(III)	solution	0.89	0.225	7.93	9.33	11.5
Tb(III)	Ho(III)	solid	1.04	0.279	7.93	9.33	10.9
Tb(III)	Er(III)	solution	0.80	0.138	3.39	8.09	11.0
Tb(III)	Er(III)	solid	0.85	0.122	3.39	8.09	11.2

[a] Calculated by using absorption spectra of $[Ln(III)(DPA)_3]^{3-}$ complexes. [b] Calculated with the parameter values $\kappa^2 = {}^2/_3$, $n^{-4} = 0.294$, $\phi_{Eu} = 0.27$, and $\phi_{Tb} = 0.48$. [c] r(X-ray) = 11.7 Å (ref 49).

different Ln(III) ions occupying distinct Ca(II) ion binding sites. This discussion will be confined to our studies on thermolysin, although investigations of parvalbumin[53] and calmodulin[56] have also been carried out.

The exchange-inert nature of Ln(III) ions occupying site S(1) of thermolysin makes it possible to substitute a luminescent ion (e.g., Tb(III) or Eu(III)) at this site and a different absorbing ion (e.g., Nd(III), Pr(III), Ho(III), or Er(III)) at sites S(3) and S(4). Nonradiative energy transfer can then be monitored by measuring the effect of the presence of energy-acceptor ions on the excited-state reciprocal lifetime of the donor ion. The experimental τ^{-1} values and the derived transfer efficiencies, E, are given in Table I for various hybrid-substituted Ln(III) derivatives of thermolysin. Owing to the feeble absorption properties of Ln(III) ions, recourse was made to model chelate absorption spectra for the evaluation of the spectral overlap integrals, J. Figure 6A shows the data necessary for the calculation of J for the Tb(III) → Nd(III) donor–acceptor pair. Unlike the case of Co(II) as an acceptor, the absorption spectra of Ln(III) complexes consist of many narrow bands, some of which are "hypersensitive"[67] to the ionic environment leading to some uncertainty in J. The donor ion quantum yield, ϕ, is also difficult to determine exactly. However, upper limits for this quantity are provided by the ratio τ_{H_2O}/τ_{D_2O}. It should be noted that a change in R_0^6 of a factor of 2 leads to distance estimates differing only by ±12%.

The Ln(III) hybrid thermolysin species listed in Table I each contain a single luminescent donor ion at S(1) and two potential acceptor ions at sites S(3) and S(4). Considering the inter metal ion distances (Figure 4) and the r^{-6} dependence of Förster-type energy transfer, it is expected that S(1) to S(3) transfer will amount to less than 1% of the S(1) to S(4) energy transfer, so the former can safely be neglected. Dissimilar ions occupying sites S(1) and S(4) are, in effect, an isolated donor–acceptor pair. The τ^{-1} values (Table I) were obtained under conditions where the concentration of Ln(III) acceptor ions free in solution was low enough to make negligible diffusion-limit, collisional deexcitation.[68,69] The measurements therefore represent pure intramolecular energy transfer.

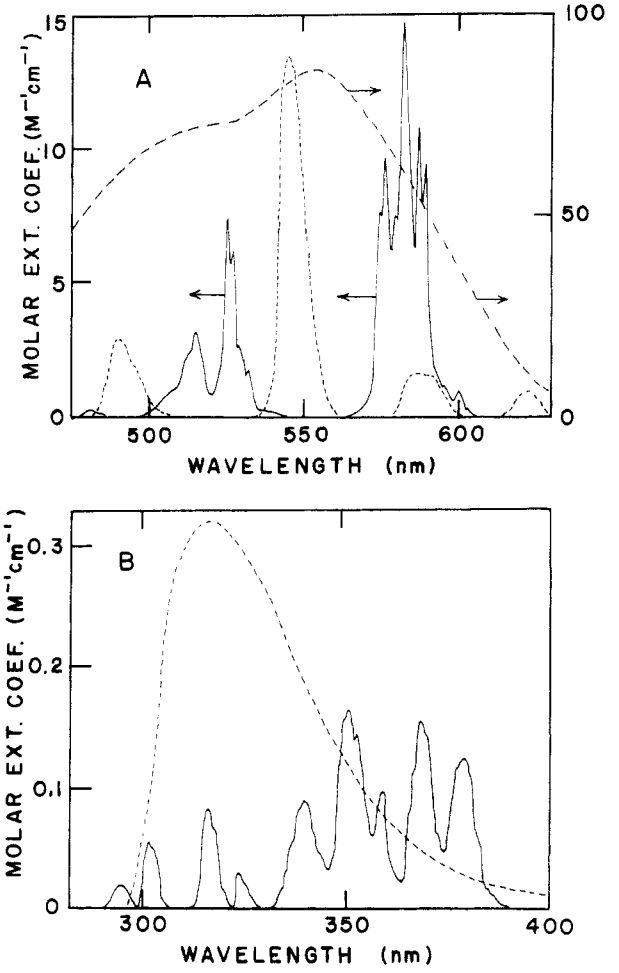

Figure 6. (A, short-dashed line) Corrected emission spectrum of Tb(III) bound to Ca site S(1) of thermolysin; (long-dashed line) absorption spectrum of Co(II) bound at the active zinc site of thermolysin; (solid line) absorption spectrum of $Nd(DPA)_3^{3-}$ complex. (B, dashed line) Corrected emission spectrum (λ_{ex} = 295 nm) of tryptophan in cod III parvalbumin; (solid line) absorption spectrum of 1:1 Tb(III)–diethylenetriaminepentaacetate complex.

The r values estimated from the energy transfer efficiencies are in good agreement with the X-ray distance of 11.7 Å between calcium sites S(1) and S(4), except

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for the Tb(III)–Nd(III) results. It is not known why the Tb(III)–Nd(III) pair appears to exhibit more energy transfer than expected, a feature also found in our study of parvalbumin.[53] It may be related to the fact that the acceptor transition ($^5G_{5/2} \leftarrow {}^4I_{1/2}$) chiefly responsible for the spectral overlap is "hypersensitive",[67] leading to an underestimation of the J overlap integral or to some contribution from a dipole–quadrupole mechanism. Experiments with nonacceptor ions (for which J integrals vanish) at sites S(3) and S(4) were carried out, and in no case was any increase in τ^{-1}, indicative of energy transfer, noted, nor was measurable energy transfer observed for Eu(III) as the donor ion with Ho(III) or Er(III) in the acceptor ion sites. This is consistent with their small calculated respective J values, 0.48×10^{-18} and 0.61×10^{-18} cm^6 mol^{-1}, and derived respective R_0 values, 5.3 and 5.5 Å.

Solid-State–Solution State Comparisons. The present technique permits a direct comparison of the properties of a macromolecule in the solution and crystalline states. Table I shows that within experimental error there are no differences in energy transfer efficiencies between the solution and solid states of thermolysin. This implies that there are no structural changes upon dissolution which significantly affect the distance between metal ion binding sites S(1) and S(3). Considering the importance of solution-state–crystalline-state comparisons in applying crystallographic results to solution-state macromolecules, this structural method, which is applicable to both states, shows particular promise.

The generally satisfactory agreement between our energy transfer measurements on the structurally well-characterized proteins thermolysin and parvalbumin substantiates our initial assumption of a dipole–dipole mechanism for inter-Ln(III) ion energy transfer and settles the controversy over the multipolar nature of the mechanism.[70-74] The protein thermolysin allows one, in effect, to isolate a pair of Ln(III) ions (the theoretically treated case), whereas experiments with doped glasses and single crystals have potential multibody interactions[73,74] as complicating factors.

Measurement of Distance between Intrinsic Protein Fluorophores and Bound Metal Ions

Many of the early investigations of the interaction of Ln(III) ions with proteins exploited in fact that protein-bound Tb(III) is often sensitized to luminesce strongly in the visible when irradiated in the region of aromatic amino acid absorption (250–300 nm). This phenomenon is quite general. In a survey of 40 proteins, Brittain et al.[75] showed that 36 exhibited sensitized Tb(III) emission. These workers demonstrated the utility of excitation spectroscopy in the UV region in identifying the aromatic amino acid (phenylalanine, tyrosine, or tryptophan) responsible for transferring energy to the Tb(III) ion. Interestingly, only Tb(III) is sensitized to emit strongly upon binding to proteins, while both Tb(III) and Eu(III) emission can be sensitized upon being liganded by nucleic acids.[76]

While this sensitization phenomenon is generally attributed to a radiationless energy transfer, the exact mechanism of the transfer had not been subjected to theoretical or experimental scrutiny prior to the investigations in this laboratory,[77,78] one of which is described here. Except for the case of coordinated tyrosine hydroxyl oxygen, where through-bond energy transfer could occur, a through-space mechanism must be operative. A Förster-type dipole–dipole mechanism is the most obvious candidate, but a dipole–quadrupole mechanism has been suggested also.[79]

The above-mentioned study[77] set out (1) to establish the energy transfer mechanism, (2) to determine why Eu(III) luminescence is not significantly sensitized, and (3) to assess the feasibility of tryptophan (Trp) to Eu(III) or to Tb(III) energy transfer distance measurements.

The quantitation of this energy transfer process was accomplished by using a parvalbumin isotype from codfish (Cod III) which contains a single tryptophan at a known position in the amino acid sequence and binds Ln(III) ions simultaneously at the primary CD and EF calcium-binding sites.[53,54,77] The sequence and X-ray data reveal that the single Trp residue is located fortuitously 11.6 Å from *both* the CD and EF metal ion binding sites. Thus the energy transfer involves a single donor and two acceptors.

When Cod III parvalbumin is titrated with various Ln(III) ions at pH 4, while Trp fluorescence is monitored (λ_{ex} = 295 nm, λ_{em} = 313 nm), no effect is observed for Tb(III) or any of the ions except for Eu(III), which causes a 75% decrease in Trp fluorescence upon addition of 2 equiv of this ion. This result implies an energy transfer efficiency, E, of 0.50 (eq 3), when corrected to correspond to a single Trp–Eu(III) pair.

On the other hand, when Cod-III parvalbumin containing 2 equiv of either Eu(III) or Tb(III) ion is irradiated at 295 nm (Trp absorption) and monitored in the regions of maximum metal ion luminescence, only Tb(III) luminesces significantly. The energy transfer to Tb(III) can be quantitated by measuring the relative number of photons emitted by Trp and Tb(III), using eq 7,[80,81] where $A_{Tb(III)}$ and A_{Trp} are the integrated areas

$$E_{obsd} = \frac{A_{Tb(III)}}{A_{Trp}} \frac{\phi_{Trp}}{\phi_{Tb(III)}} \tag{7}$$

of luminescence emission (on a cm^{-1} scale) of Tb(III) and Trp, respectively, and $\phi_{Tb(III)}$ and ϕ_{Trp} are the respective quantum yields. After correcting to the single Trp → Tb(III) case, an efficiency, E, of 5.32×10^{-4} was determined experimentally. This small value for E produces easily observable sensitized luminescence from Tb(III) but no measurable quenching of Trp fluorescence.

Figure 6B shows the emission spectrum of Trp superimposed on the absorption spectrum of a Tb(III)

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model complex. Values for the J overlap integrals ranging from 0.39×10^{19} to 0.69×10^{19} cm^6 mol^{-1} were obtained for various model systems. These lead to R_0 values averaging 3.35 Å. This R_0 value together with the measured transfer efficiency predicts a Trp–Tb(III) separation of 11.8 Å, in excellent agreement with the distance estimated from X-ray and sequence information (11.6 Å). This result supports the initial assumption of a Förster-type dipole–dipole mechanism and demonstrates the utility of the method for making distance measurements.

The contrasting behavior of Eu(III) (marked quenching of Trp fluorescence; insignificant sensitized emission) arises because of a low-lying ligand to Eu(III) charge transfer absorption band.[82-85] This band has been measured for Eu(III) bound to parvalbumin isotype carp-III (which contains no Trp) and is found to yield a much larger spectral overlap integral and, of course, a larger R_0 value (~10.2 Å). Judging from the behavior of model complexes, this charge transfer band can be expected to vary markedly both in position and in intensity from system to system. For this reason quenching of Trp fluorescence by Eu(III) is expected to be much less reliable than quantitated Tb(III) emission for the purpose of making distance estimates. The lack of sensitized emission in the case of Eu(III) arises from the fact that the charge transfer band correlates with a f^7 excited state so no emission from the $^5D_0$ term of the f^6 configuration is to be expected. Thus while energy is efficiently transferred to Eu(III), it is virtually all radiationlessly dissipated and does not appear as luminescence.

In the case of Eu(III) bound to nucleic acids, the fluorescent nucleotide 4-thiouridine has been implicated as the energy donor.[76] This fluorophore emits at energies much lower than tryptophan and well separated from any charge transfer state. For nucleic acids both Eu(III) and Tb(III) exhibit strongly sensitized emission.

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Concluding Remarks

In addition to the types of information discussed above, laser-induced luminescence experiments are capable of providing a detailed knowledge of the ligand field at the metal ion binding site. We recently showed, using the 488-nm line of a CW argon ion laser to excite the $^5D_4$ emissive state of Tb(III), that environmentally sensitive fine structure can be measured on several emission bands, especially the $^5D_4 \rightarrow {}^7F_5$, $\rightarrow {}^7F_4$, and $^7F_3$ transitions.[86] Tb(III) is less than ideal for this purpose since both the initial and final states involved in the transitions are split by the ligand field. This difficulty is not encountered in the case of Eu(III) where both the ground ($^7F_0$) and emissive ($^5D_0$) states are nondegenerate. Preliminary work[87] with Eu(III) shows that ligand field splittings can be directly measured on the excited $^5D_1$ and $^5D_2$ levels by excitation spectroscopy and on the various J components of the ground ^7F term when the emissions originating from the $^5D_0$ excited state are examined under moderately high resolution (0.3 nm). A structure-sensitive characterization of individual metal ion binding sites can be achieved using these methods.

In summary, this Account has outlined various classes of Ln(III) ion luminescence probe experiments and the types of information provided by each. Most of the experiments are possible only through the use of laser excitation. These techniques are sensitive, nondestructive, and applicable to a wide variety of biological macromolecules.

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