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Time-Resolved Europium(III) Excitation Spectroscopy: A Luminescence Probe of Metal Ion Binding Sites

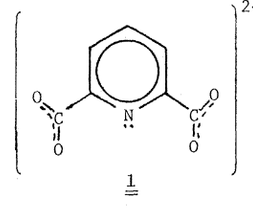
Abstract. *A laser-induced luminescence technique is introduced for probing the structure and equilibria of lanthanide complexes and lanthanide ion binding to macromolecules. The method involves the excitation of the* $^7F_0 \rightarrow {}^5D_0$ *transition between nondegenerate levels in the europium(III) ion by means of an intense pulsed dye laser source. Excitation profiles obtained by scanning the laser through the transition region reveal distinct peaks characteristic of individual europium(III) ion environments. The technique may be used to characterize the species present in complex equilibria in solution or to study europium(III) binding to macromolecules. Distinct europium(III) binding sites in thermolysin with long and short excited state lifetimes are observed.*

The ability of trivalent lanthanide ions, Ln(III), to serve as replacement probes for Ca(II) in calcium-binding proteins is well established (*1*-*6*). An especially promising property is the ability of several members of the series to luminesce in fluid solution at room temperature. We have shown (*7*) that useful luminescence emission can be observed at Ln(III) concentrations as low as 1 μM when an intense pulsed dye laser source is used for direct excitation of metal ion levels. Determinations of the reciprocal excited state lifetime, τ^{-1}, in both H_2O and D_2O solution provide a direct measure of the number of water molecules coordinated to a Ln(III) ion, which is itself bound to a macromolecule (*7*).

In this report we discuss a technique that is akin to absorption spectroscopy but that amplifies the sensitivity of the absorption experiment by several orders of magnitude. This is accomplished by monitoring absorption indirectly by means of emitted photons. Excitation spectroscopy with an intense, rapidly pulsed laser excitation source allows us to record absorption profiles of protein-bound Ln(III) ions with absorption coefficients, ε, as low as $0.01 M^{-1}$ cm^{-1} down to concentrations of 0.1 mM and below.

Our present concern is with the $^7F_0 \rightarrow {}^5D_0$ transition in Eu(III) at $\sim$ 17,250 cm^{-1} (580 nm), which has certain unique advantages. Since both the initial and final states are nondegenerate in the free ion, neither can be split by a ligand field, and thus a single sharp transition is to be expected for a particular Eu(III) ion. The energy of the $^7F_0 \rightarrow {}^5D_0$ transition (hereafter referred to as the 0-0 transition) is slightly dependent on the environment of the Eu(III) ion; and this transition is observed to range over slightly more than 1 nm for the systems we have investigated (1 nm $\simeq$ 30 cm^{-1} at 580 nm). The 0-0 transition is an extremely weak one ($\varepsilon \simeq 0.01 M^{-1}$ cm^{-1}) (*8*) and its study by absorption spectroscopy in dilute protein solutions is out of the question. Our experiment is performed, as described below, with much of the same apparatus employed for lifetime measurements (*7*). The Eu(III)-containing sample is irradiated with light (at or near 580 nm) from a pulsed (10 Hz) dye laser pumped by a nitrogen laser. The emission monochomator is set with wide slits to accept the intense $^5D_0 \rightarrow {}^7F_2$ emission at $\sim$ 612 nm. The excitation source, whose spectral bandwidth is approximately 0.01 nm, is then scanned through the 0-0 transition region. The intensity of the signal detected is proportional, among other factors, to the number of photons absorbed by this transition. For a single complex in solution, perfect Lorentzian peaks are observed with widths at half height of $\sim$ 8 cm^{-1}. They are thus sharp enough to be able to reflect fairly small changes in transition energy in going from one Eu(III) ion environment to another. Barring fortuitous coincidences, one observes as many 0-0 transitions as there are Eu(III) environments in the sample.

Our method may be applied to the study of complex equilibria in solution. Figure 1A shows the excitation profiles of the 0-0 transition during the course of the titration of Eu(III) (present as the chloride salt) with DPA^{2-} (dianion of dipicolinic acid, **1**, present as the sodium



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salt) in solutions with an adjusted pH of 6.0. As the DPA^{2-}/Eu(III) ratio is increased, marked changes occur in the spectra. Four distinct peaks are observed at various stages in the titration. On the basis of lifetime measurements, these correspond to the aqua ion: Eu_{aq}^{3+}, $[Eu(DPA)]^+$, $[Eu(DPA)_2]^-$, and $[Eu(DPA)_3]^{3-}$, which occur at progressively lower energies (Table 1). The energy differences are small, varying from 9 to 17 cm^{-1} between adjacent species, but with widths at half height of about 8 cm^{-1} the individual species are readily detectable.

The intensity of an individual peak in an excitation spectrum is determined by the product of the absorption coefficient for the band of the species in question and the quantum yield of luminescence of the emitting species. In the present experiment, signals of the Eu_{aq}^{3+} ion and the $[Eu(DPA)_3]^{3-}$ ion require much higher excitation intensities and signal ampli-

Table 1. Energies, excited-state lifetimes in H_2O and D_2O, and estimated numbers of Eu(III)-coordinated water molecules for the Eu(III)-dipicolinate system and Eu(III)-substituted thermolysin.

Species	Energy (cm^{-1})	τ_{H_2O} (μsec)	τ_{D_2O} (μsec)	q
Eu_{aq}^{3+}	17,272	104	2630	9.6
$[Eu(DPA)]^+$	17,263	169	2966	6.3
$[Eu(DPA)_2]^-$	17,248	304	3172	3.1
$[Eu(DPA)_3]^{3-}$	17,231	1640	3153	0.3
Thermolysin				
Site 1	17,253	555	1650	1.2
Sites 3 and 4	17,265	242	908	3.2

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fication than do those of the intermediate species, $[Eu(DPA)]^+$ and $[Eu(DPA)_2]^-$. The vertical scales of the lowermost and uppermost traces in Fig. 1A have therefore been greatly expanded for illustrative purposes. The lower sensitivity toward excitation of the Eu_{aq}^{3+} and $[Eu(DPA)_3]^{3-}$ ions results in part from their greater symmetry and concomitantly smaller absorption coefficients (*9*). In addition, Eu_{aq}^{3+} has a much lower quantum yield (0.019) than the other species because there are nine to ten water molecules in its first coordination sphere. The excitation spectrum of this ion may be recorded with lesser difficulty in D_2O solution because of the much higher quantum yield (0.78) for the aqua ion in this solvent (*8*). These findings show that one must be cautious when carrying out studies of this type. Not all species, even closely related ones, can be detected with the same sensitivity. A plot (not shown) of the signal intensities of the various species as a function of the DPA^{2-}/Eu(III) ratio reveals titration curves consistent with equilibrium constants for this system obtained by other means (*10*).

An extremely important aspect of this particular experiment is that each excitation peak is, in effect, labeled by the excited state lifetime of the emitting species. Thus each of the complexes present during the course of the titration has its own characteristic excited state lifetime, τ. The values of τ are listed in Table 1 for titrations carried out in H_2O and D_2O. For the titration in H_2O there is the expected dramatic increase in τ values as the chelation of each successive DPA^{2-} ligand displaces additional water molecules from the first coordination sphere. By applying the quantitative relationship that we developed earlier (*7*) between $\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1}$ and the number of coordinated water molecules, q, in the first coordination sphere of the Eu(III) ion (*11*), we obtained the q values listed in Table 1. The results are in accord with the expectation that each tridentate DPA^{2-} ligand displaces three coordinated water molecules.

It is appropriate here to consider the relationship between the excited state lifetimes and the time scales of any chemical exchange or interconversion processes that may be occurring in solution. If such processes occur in times short compared to τ, then any particular Eu(III) ion in its excited state will be shuttled many times between different chemical environments. Individual excitation peaks will still be observable for the absorbing species, but all will exhibit the same τ value, which represents a weighted average of the τ values that would be characteristic of the individual species in the absence of exchange. The exchange rates in the Eu(III)-DPA^{2-} sys-

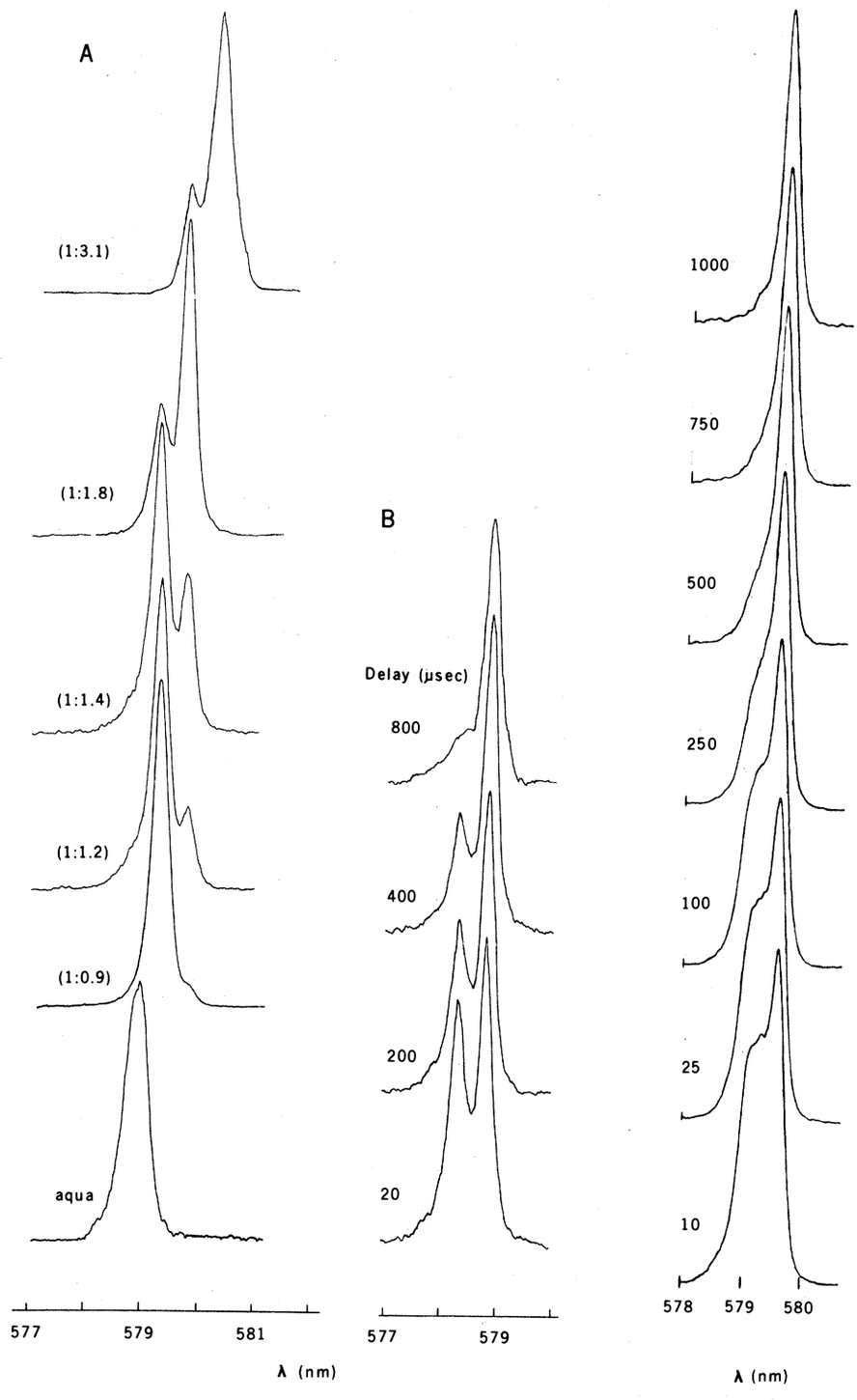

Fig. 1. (left). (A) Successive excitation spectra appearing during the course of the titration of Eu(III) with DPA^{2-} (bottom to top). Individual peaks due to the aqua ion and the 1:1, 1 : 2, and 1 : 3 Eu(III)-DPA^{2-} complexes are apparent. The metal-to-ligand ratio is recorded to the left of each spectrum. The excitation spectrum of the aqua ion was recorded in a 0.05*M* D_2O solution. (B) Time resolution of the spectra of $[Eu(DPA)]^+$ and $[Eu(DPA)_2]^-$ for a solution with a molar ratio of Eu(III) to DPA^{2-} of 1:1.7. The boxcar delay time is indicated to the left of each spectrum. Fig. 2 (right). Time-resolved spectra of thermolysin (0.3 m*M*, tris buffer, *p*H 7.5) containing three equivalents of Eu(III). The bottom trace shows the spectrum recorded 10 μsec after the laser pulse, and that at the top shows the spectrum recorded 1000 μsec after the laser pulse. The temporal resolution of the spectrum of the site containing the long-lived Eu(III) from the spectra of the site or sites containing the short-lived Eu(III)'s is apparent from the intervening spectral traces.

tem are slow, as might be expected for a rigid tridentate ligand; however, we have observed other cases where the rapid exchange limit obtains. In the case of such rapid exchange any attempt to achieve temporal resolution in the excitation spectrum, such as that described below, will fail. The excitation experiment thus has the potential for yielding qualitative and perhaps quantitative information regarding chemical exchange and interconversion processes.

In systems with multiple Eu(III) ion environments, each with a characteristic lifetime, it is possible to record excitation spectra in a time-resolved mode, provided the individual lifetimes are sufficiently different. The signal from the photomultiplier tube, after amplification and suitable conditioning, is fed into a boxcar signal averager. The boxcar samples the decay curve for a preset time interval and delay following the trigger pulse. For example, if the sample contains two species, one with a short τ and one with a long τ, setting the boxcar delay very close to the trigger (coincident with the laser pulse) will result in luminescence emission from both long- and short-lived species being recorded. If a longer delay is set, the short-lived species will have decayed away before the averager is activated and only the spectrum of the long-lived species will be recorded. This is illustrated for the DPA^{2-}-Eu(III) system in Fig. 1B. At a DPA^{2-}/Eu(III) ratio of 1.7 significant quantities of both $[Eu(DPA)]^+$ and $[Eu(DPA)_2]^-$ exist in equilibrium. The τ for $[Eu(DPA)]^+$ (169 μsec) is sufficiently shorter than the τ for $[Eu(DPA)_2]^-$ (304 μsec) that as the boxcar delay time following the laser excitation pulse is increased from 20 to 800 μsec (ascending Fig. 1B), the spectrum of the former ion disappears almost completely. Such temporal resolution experiments may be useful for simplifying complex excitation spectra that are due to solution equilibria or the presence of multiple metal binding sites in macromolecules.

To assess the usefulness of time-resolved Eu(III) excitation spectroscopy (TREES) for the study of Eu(III) binding to macromolecules, we applied this technique to the structurally well-characterized (*12*) zinc endoprotease thermolysin, which binds one Zn(II) and four Ca(II) ions in the native state. This enzyme is known (*13*) to bind a Ln(III) ion strongly at calcium site 1 of a double site when Ln(III) ions are added to solution, even in the presence of Ca(II). Furthermore, it has been shown (*6*) by x-ray crystallographic techniques that soaking crystals of thermolysin in the presence of Ln(III) ions under specified conditions results in the isomorphous replacement of Ca(II) by Ln(III) at three distinct binding sites (sites 1, 3, and 4). The spectrum of thermolysin to which three equivalents of Eu(III) are bound was recorded in a time-resolved mode and is shown in Fig. 2. The lowest trace in Fig. 2 was recorded with a short (10 μsec) delay after the laser trigger. It appears to consist of a strong peak at a low energy with a feature, probably consisting of two peaks, at a higher energy. As the delay time is progressively increased (ascending Fig. 2), the high-energy feature becomes progressively less and less prominent, and it is virtually undetectable at a delay time of 1000 μsec. This uppermost trace belongs to a long-lived Eu(III) species and is identical to the excitation spectrum obtained from samples of thermolysin to which less than one equivalent of Eu(III) was added; it therefore corresponds to Eu(III) in Ca(II) site 1. The short-lived peaks at higher energies are consequently assigned to Eu(III) bound at sites 3 and 4. The energies and individual τ values measured in H_2O and D_2O solutions are recorded in Table 1, along with the estimated numbers of coordinated water molecules. These results are in reasonable accord with the crystallographic findings (*3*) that the Eu(III) ion at Ca(II) site 1 has one coordinated water molecule, while the Eu(III) ions at sites 3 and 4 have three each.

These initial results suggest that the TREES technique has considerable potential for elucidating the details of Eu(III) binding to macromolecules. Questions regarding the characterization of distinct binding sites, sequential binding, the numbers of metal-coordinated water molecules, complex equilibria in solution, and even chemical exchange processes may be addressed by this method. The technique should be particularly valuable for applications requiring selective excitation of ions in specific sites—for example, to monitor Förster-type energy transfer from a particular bound Eu(III) ion to a bound energy acceptor ion (*13*).

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The Dielectric Constant of Phospholipid Bilayers and the Permeability of Membranes to Ions

Abstract. *The Born charging equation predicts that the permeability of a phospholipid bilayer membrane to ions should depend markedly on the dielectric constant of the membrane. Increasing the dielectric constant of an artificial bilayer increases its permeability to perchlorate or thiocyanate by a factor of 1000, to a value comparable to that of mitochondrial membranes.*

The permeability of the inner mitochondrial membrane to thiocyanate and perchlorate ions (*1*) is three to four orders of magnitude higher than the permeability of decane-containing lipid bilayers to these ions (*2*), even when the bilayers are formed from mitochondrial lipids (*3*). This disparity could be due to the presence of proteins in the biological membrane, which could increase the dielectric constant of the lipid bilayer. We present evidence that the dielectric constant of artificial lipid bilayers can be increased with 1-chlorodecane, and show that this increase in dielectric constant enhances the perchlorate or thiocyanate permeability of lipid bilayers to a value commensurate with that of mitochondrial membranes.

We assume the bilayer to be an iso-

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