Lanthanide Ion Probes of Structure in Biology. Laser-Induced Luminescence Decay Constants Provide a Direct Measure of the Number of Metal-Coordinated Water Molecules

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Abstract: Direct excitation of f-electron levels of Eu(III) and Tb(III) using a pulsed dye laser source results in luminescence emission which decays exponentially with an environmentally sensitive decay constant (reciprocal lifetime). The presence of OH oscillators in the first coordination sphere of the metal ion provides an efficient pathway for radiationless deexcitation via energy transfer to OH vibrational overtones. This pathway is virtually eliminated upon replacement of OH by OD oscillators. Decay constant measurements on both H_2O and D_2O solutions, or upon crystalline solids containing these molecules, allow the determination of the number of water molecules coordinated to the metal. The validity of the method is established by experiments on a variety of crystalline complexes of known X-ray structure involving from zero to nine coordinated water molecules. The technique is applied to a number of model chelate systems in solution and to the structurally well-characterized proteins thermolysin and parvalbumin. There is close correspondence between the solution and solid-state results. A particular advantage of the present method lies in the extreme simplicity of the experimental technique and in its applicability to dilute protein solutions.

Metal ion binding sites of macromolecules provide a valuable point of focus for a variety of spectroscopic and magnetic resonance experiments designed to probe structure-function relationships in proteins and nucleic acids. Even when the native ion is devoid of most useful reporter-ion properties, e.g., the colorless, diamagnetic Mg(II), Zn(II), and Ca(II) ions, it is quite often possible to replace the native ion with a substitutional probe species suitable for particular physicochemical experiments. Classic examples of such procedures include the substitution of Mn(II) (an NMR relaxation and EPR probe) for Mg(II),[1] Co(II) (an EPR and absorption spectral probe) for Zn(II),[2] and the trivalent lanthanide ions, Ln(III), for Mg(II) in nucleic acids or Ca(II) in proteins.[3,4] The present paper will concentrate on the use of Ln(III) ions as substitutional probes for Ca(II) in proteins. Isomorphous replacement of Ca(II) by Ln(III) has been demonstrated by protein X-ray crystallography in two cases;[5,6] furthermore, Ln(III) ions have been shown to activate a number of proteins and enzymes to their biological function in the stead of Ca(II).[7-12] In other instances Ln(III) ions act as inhibitors of the Ca(II) or Mg(II) function.[13-15]

This paper will focus on the luminescence properties of two members of the Ln(III) series, namely, Eu(III) and Tb(III). While the fluorescence of organic moieties has been greatly exploited in biochemical research, metal ion luminescence remains relatively undeveloped as a technique. Reasons for this include the fact that of all of the metallic cations in the periodic table, only certain members of the Ln(III) series are capable of luminescence emission in fluid solution at room temperature, when present as the aqua ions or as complexes of simple oxygen donor ligands. It is also true that the luminescence of Ln(III) ions is extremely weak when compared to organic fluorophores. This weakness in luminescence arises principally from the low oscillator strength ($\sim 10^{-6}$) of their absorption bands. This makes it difficult, using ordinary fluorimetry, to achieve sufficient excited-state populations for the study of these ions in necessarily dilute biological systems. In certain, but by no means all, cases the inherent weakness of Ln(III) ion luminescence is overcome by an energy transfer from an absorbing aromatic amino acid residue of the protein to a bound Tb(III) ion. This energy transfer, when it occurs, has the effect of greatly enhancing the intensity of metal ion luminescence emission. Alternatively, this limitation (weakness of absorption) can be overcome by using the large photon flux of a laser as the excitation source.[16]

An earlier report from this laboratory[16] established that luminescence emission can be observed on quite dilute Eu(III) and Tb(III) solutions using a pulsed dye laser excitation source. By employing visible radiation ($\lambda > 450$ nm) for the direct excitation of metal ion levels, problems of protein absorption or photosensitivity to UV light are eliminated. We further demonstrated the environmental sensitivity of the measured excited state exponential luminescence decay constants (reciprocals of the excited-state lifetimes). In particular we proposed that such measurements represent a simple and fairly accurate method of determining the number of water molecules coordinated to a Ln(III) ion which itself is bound to a protein or coordinated to other ligands. While the results of our initial studies on solutions of model systems are quite reasonable and in accord with chemical intuition, there exists no independent experimental means with which to verify the solution results. It is the purpose of the present paper to develop the technique further and to verify the method by examining the results of experiments performed on structurally well-characterized crystalline solids. The close correspondence between data obtained on solutions and the results on solids containing complexes involving known numbers of coordinated water molecules establishes the validity of the method. In addition, results on the protein parvalbumin and the enzyme thermolysin, for which the details of the Ln(III) ion binding have been established via X-ray crystallography,[6,7] further substantiate the utility of our experiment. The applicability of our experimental technique to solid-state and heterogeneous samples demonstrates the feasibility of studying membrane-bound ions as well as for establishing the correspondence between solution and solid-state structures.

Experimental Section

Pulsed Dye Laser and Associated Optics and Electronics. For the luminescence decay constant determinations a Phase-R Model DL-2100B (Phase-R Co., Durham, N.H.) coaxial-flash lamp-pumped dye laser was employed. The width of the laser pulse is typically ~ 0.5 μs. Ethanol solutions of a variety of coumarin dyes (Exciton Chemical Co., Dayton, Ohio) were used, the most satisfactory ones being LD490 (λ_0 488 nm, Tb(III)), Coumarin 460 (λ_0 461 nm, Eu(III)), and LD473 (Tb(III) and Eu(III)). Tuning of the lasing region of the dye

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was accomplished by a single intracavity prism which allowed for extracting a 1-nm bandwidth from the bandwidths of 35-70 nm of the different dyes. Pulse energies were typically 100 mJ-3 J.

The laser light was focused by a short focal-length lens onto the powdered solid sample which was placed on a stage or focused into a standard 1-cm fluorimetry cell for solution samples. The light emitted from the lanthanide samples was collected at 90° by a $f/0.95$ 50-mm camera lens, focused onto the slits of a JY-Optical H-20, 0.2-m monochromator (Instruments SA, Inc. Metuchen, N.J.) and passed through an orange OG-515 long-pass colored glass filter (Schott Glass, Inc., Durea, Pa.). When studying dilute solutions the entire sample chamber and collection optics were encased in a box containing suitable baffles and an entrance port holding appropriate short-pass filters (Ditric Optics, MA) through which the laser pulse passed. This arrangement was necessary when looking at low signal levels so as to suppress both the background and the flashlamp (white) light.

Lanthanide ion luminescence was detected by a Hamamatsu HTV-R928 photomultiplier tube (PMT) followed by dc amplification. For the lifetimes being measured it was found unnecessary to gate the high voltage on the dynode chain of the PMT. Instead, a Zener diode, placed in a feedback loop of the amplifier, clipped and diverted to ground the fast overload created by the unavoidable initial scatter from the laser pulse. An effective recovery time constant of <0.4 μs was achieved. Transient effects such as photocathode saturation reported by others[17,18] did not interfere with either the short-term stability or the long-term sensitivity of the PMT nor did they interfere with obtaining stable, reproducible signals.

Following dc amplification, the transient signal was fed into a Biomation Model 805 waveform recorder in the dual timebase mode (maximum A/D conversion rate 5 MHz). After digitalization the analog waveform was reconstructed at a suitable rate for readout onto either a strip chart or X-Y recorder.

Semilogarithmic plots of the luminescence intensity vs. time were made and the luminescence decay constants, k_{obsd}, were thus extracted.

Materials. All lanthanide salts were obtained from either Research Organic/Inorganic or Alfa-Ventron Chemicals. Organic ligands were obtained as indicated in the footnotes of Table I. Deuterium oxide (99.7%) was obtained from Merck Isotopes, and CH_3CH_2OD (99.8%), NaOD (99.0%), and DCl (99.0%) were obtained from Strohler Isotopes. During the preparation of crystalline samples containing D_2O the beakers containing the growing crystals were placed in a desiccator suitably modified so as to allow a steady stream of dry N_2 gas to flow through the chamber. The N_2 was redundantly scrubbed of residual H_2O by passing it through the series of desiccants: $CaCl_2$, NaOH, P_2O_5, and H_2SO_4. The growth chamber was kept at an ambient 30 °C.

To monitor any H_2O contamination which occurred during the growing period, aliquots of the mother liquor were extracted and placed in 5-mm NMR tubes. The integrated area of the ^1H_2O (or the $^1HOC_2H_5$ for the acetylacetonate and terpyridine preparations) signal was recorded on a Varian A-60A NMR spectrometer. Each mother liquor sample was then purposely and successively contaminated with small known amounts of light water and reintegrated. The integrated signal intensity is linearly related to the ^1H_2O concentration; hence, by back-extrapolating an intensity vs. concentration plot to zero intensity the original H_2O contamination of the D_2O mother liquor was determined.

With the exceptions of the oxalates and acetylacetonates, which were prepared in deuterated solvents, all of the D_2O variety of the lanthanide hydrates were made by redissolving the H_2O species in a sufficient volume of D_2O so as to statistically minimize the resultant H_2O contamination. Samples whose mother liquor ^1H_2O resonance intensity resulted in excess of 5% H_2O contamination were recycled.

X-ray Powder Diffraction. Powder diffraction data of all the compounds listed in Table I were recorded on a Norelco X-ray diffractometer using a standard $2° - 2\theta$/min scan rate. A Picker scintillation counter and associated electronics recorded the reflections. To circumvent X-ray fluorescence of the lanthanides Fe Kα radiation employing a MnO_2 β filter was used.

Since powder diffraction data have been recorded for only a few of the substances studied, calculated patterns were generated using a computer program named POWD.[19] This routine calculates a theoretical powder diffraction pattern from input single-crystal structural data. Included in Table I are only those substances whose single crystal structures have been determined, and which are isomorphous with their Tb and Eu analogues. Moreover, those substances which possess other than a single molecular species within their unit cells were eliminated from study. Only those samples possessing a 1:1 match between observed and calculated diffraction patterns are included in this report.

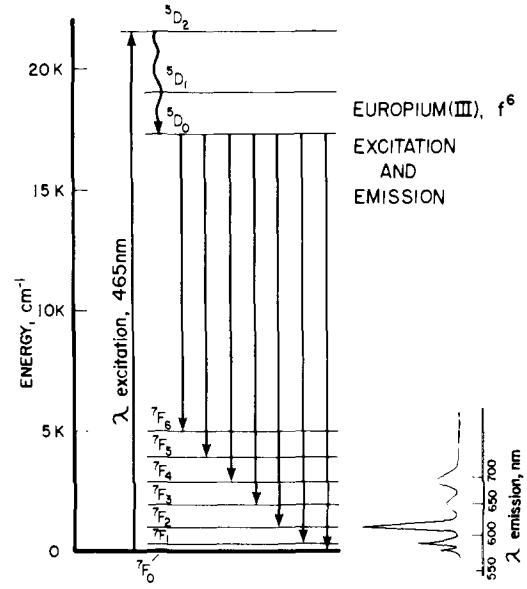

Figure 1. Electronic energy scheme for Eu(III). Laser-induced excitation of the $^5D_2$ state is followed by rapid (<5 μs) nonradiative relaxation (ref 39) to the luminescent $^5D_0$ state (wavy line). Emission to the ground ^7F manifold from the $^5D_0$ state is indicated by the heavy arrows with a "typical" intensity profile of the emission bands depicted in the figure to the right.

Results

A variety of crystalline Eu(III) and Tb(III) complexes of known crystal structure were prepared by literature methods as indicated in the footnotes of Table I.[20-38] The substances chosen for study had varying numbers of water molecules in the range zero to nine coordinated to the Ln(III) ions. Two series of crystals were prepared, one containing coordinated H_2O and the other coordinated D_2O. The Eu(III) complexes were excited to the $^5D_2$ state by a laser flash which resulted principally in emission from the $^5D_0$ excited state as depicted in the energy level diagram of Figure 1. In the case of the Tb(III) complexes the laser light directly excited the emissive $^5D_4$ level as indicated in Figure 2. The intensity of the laser-induced luminescence was observed to decrease exponentially for all systems studied with the exponential decay constants, k_{obsd}, reported in Table I. For comparison purposes, the few relevant data on crystalline solids to be found in the literature[39-41] are also included in Table I. Table II presents the results of measurements made on solutions including those obtained on the enzyme thermolysin and the protein parvalbumin to which 1 equiv of Ln(III) ion had been added.

Discussion

Earlier work has established that a weak vibronic coupling of Ln(III) ion excited states with the OH oscillators of coordinated water molecules provides a facile path for radiationless deexcitation of Ln(III) ions.[42-50] All indications are that the OH oscillators act independently and that the rate of deexcitation via this process is *directly proportional to the number of OH oscillators in the first coordination sphere.*[47,49,50] To a reasonably good approximation the energy transfer to higher O-H vibrational overtones is independent of the constitution of the remainder of the coordination sphere of the Ln(III) ion. Importantly, this radiationless deexcitation path exhibits a very

Table I. Preparation, Characterization, and Luminescence Decay Constants for Hydrated Crystalline Solids

substance	number of water molecules in first coordination sphere	coordination number of Ln(III)	luminescence decay constant, ms^{-1} Eu(III) $k_{obsd}^{H_2O}$	Eu(III) $k_{obsd}^{D_2O}$	Tb(III) $k_{obsd}^{H_2O}$	Tb(III) $k_{obsd}^{D_2O}$	footnotes
$[Ln(H_2O)_9](ethylsulfate)_3$	9	9	9.35	0.61	2.37	0.37	*a*
$[Ln,Y(1:10)(H_2O)_9](ethylsulfate)_3$	9	9	8.85	*q*	2.33	*q*	*b*
$[Ln,Y(1:30)(H_2O)_9](ethylsulfate)_3$	9	9	8.62	*q*	2.42	*q*	*b*
$[Ln(H_2O)_9](ethylsulfate)_3$	9	9	9.01	1.33	2.40	0.66	*b*
$[Ln(H_2O)_9](bromate)_3$	9	9	*q*	*q*	2.38	0.40	*c*
$[Ln(H_2O)_9](bromate)_3$	9	9	8.69	*q*	2.37	*q*	*d*
$[Ln(H_2O)_6Cl_2]Cl$	6	8	8.25	0.71	2.08	0.53	*e*
$[Ln(H_2O)_6Cl_2]Cl$	6	8	8.33	*q*	2.07	*q*	*b*
$[Ln(H_2O)_6Cl_2]Cl$	6	8	7.69	0.61	2.06	0.42	*f*
$[Ln(H_2O)_6Cl_2]Cl$	6	8	8.19	*q*	2.07	*q*	*d*
$[Ln,Y(1:10)(H_2O)_6Cl_2]Cl$	6	8	8.47	*q*	2.07	*q*	*b*
$[Ln,Y(1:30)(H_2O)_6Cl_2]Cl$	6	8	8.54	2.63	2.07	0.91	*b*
$[Ln(H_2O)_5(Cl)(terpyridyl)]Cl_2 \cdot 3H_2O$	5	9	4.99	0.63	3.38	2.10	*g*
$[Ln_2(H_2O)_8(sulfate)_3]$	4	8	4.79	0.58	1.39	0.40	*h*
$[Ln_2(H_2O)_8(sulfate)_3]$	4	8	4.90	*q*	1.39	*q*	*b*
$[Ln_2(H_2O)_8(sulfate)_3]$	4	8	5.26	*q*	1.38	*q*	*d*
$[Ln(H_2O)_4(thiodiacetate)]Cl$	4	9	5.35	1.30	1.84	0.77	*i*
$Na[Ln(H_2O)_3(EDTA)] \cdot 5H_2O$	3	9	2.90	0.56	1.15	0.44	*j*
$\{Ln_2(H_2O)_6(oxalate)_3\} \cdot 4H_2O$	3	9	3.53	0.76	1.27	0.59	*k*
$\{(Ln,Gd(1:100))_2(H_2O)_6(oxalate)_3\} \cdot 4H_2O$	3	9	3.59	*q*	1.22	*q*	*k*
$\{Ln(H_2O)_2(NTA)\} \cdot H_2O$	2	9	2.72	0.53	0.94	0.49	*l*
$\{Ln,Gd(1:100)(H_2O)_2(NTA)\} \cdot H_2O$	2	9	2.65	*q*	0.87	*q*	*l*
$[Ln(H_2O)_2(acac)_3] \cdot H_2O$	2	8	3.10	1.00	1.33	0.90	*m*
$[Ln(H_2O)_2(nicotinate)_3]_2$	2	8	2.49	1.02	1.14	0.70	*n*
$\{Ln(H_2O)_2(isonicotinate)_3\}$	2	8	2.80	0.97	1.18	0.75	*o*
$Na_3[Ln(dipicolinate)_3] \cdot 15H_2O$	0	9	0.77	0.72	0.83	0.82	*p*

[a] This work; X-ray powder diffraction (XRD) patterns compared with Joint Committee on Powder Diffraction Standards (JCPDS) file no. 14-790 for the isomorphous holmium complex. Preparation followed that of ref 26. Barium ethylsulfate was obtained from Pfaltz & Bauer Co. [b] Reference 39. [c] This work; XRD powder patterns compared with JCPDS file no. 18-1148 for the isomorphous samarium complex. Preparation followed that of ref 26. Barium bromate was obtained from Pfaltz & Bauer Co. [d] Reference 41. [e] This work; XRD powder patterns compared with JCPDS file no. 20-69 for the isomorphorus gadolinium complex. Crystal structure: ref 20. [f] Reference 40. [g] This work; XRD powder patterns calculated from the data of isomorphous praseodymium complex. Crystal structure analysis: ref 21. Preparation followed the procedure outlined in ref 22. The 2,2′,2″-terpyridine ligand was obtained from Pfaltz & Bauer Co. [h] This work; XRD powder pattern compared with JCPDS file no. 22-1105 for the isomorphous gadolinium complex. Crystal structure: ref 23 and 24 for the isomorphous praseodymium and samarium complexes, respectively. Preparation by recrystallization of the commercial product (Alfa, Ventron). [i] This work; XRD powder pattern calculated from the data of the isomorphous neodymium complex. Crystal structure and preparation scheme: ref 25. The thiodiacetate ligand was obtained from Pfaltz & Bauer Co. [j] This work; XRD powder pattern calculated from single crystal data of the terbium complex, ref 27. Preparation followed that described in ref 27. H_4EDTA was obtained from Eastman Chemicals. [k] This work; XRD powder pattern compared with JCPDS file no. 22-487. In addition, diffraction pattern was calculated using the single-crystal structural data of the isomorphous gadolinium complex, ref 28 and 29. Preparation followed that outlined in ref 30. Oxalic acid was obtained from Eastman Chemical Co. [l] This work; XRD powder patterns compared with the calculated patterns of the non-isomorphously crystallizing praseodymium and dysprosium complexes. Crystal structure analyses: ref 31 and 32. Both europium and terbium complexes crystallized in the nine-coordinate praseodymium form. Preparation followed that of ref 33. Nitrilotriacetic acid (NTA) was obtained from Eastman Chemical Co. [m] This work; XRD powder pattern calculated from the crystal data of the europium complex, ref 34. Preparation followed that of ref 35. 2,4-Propanedione (Hacac) was obtained from Aldrich Chemical Co. [n] This work; XRD powder pattern calculated from the single-crystal data of the isomorphous samarium complex, ref 36. Preparation followed that described in ref 37. Nicotinic acid was obtained from Eastman Chemical Co. [o] This work; XRD powder pattern calculated from the single-crystal data of the isomorphorous lanthanum complex, ref 38. Preparation followed a scheme similar to that in ref 37. Isonicotinic acid was obtained from Eastman Chemical Co. [p] This work; XRD powder pattern calculated from the single-crystal data of the isomorphous neodymium complex, ref 40. The preparation followed that outlined in ref 63. Dipicolinic acid was obtained from Aldrich Chemical Co. [q] Not reported. [ ] brackets indicate the coordination unit. { } brackets indicate the stoichiometry of a polymeric structure.

large isotope effect such that O–D oscillators are much less efficient in effecting this deexcitation process.[42-50] This isotope effect allows us to identify the contribution of coordinated H_2O to the rate of deexcitation by the simple expediency of replacing H_2O by D_2O. A schematic diagram of the situation for the Tb(III) ion is shown in Figure 3. The observed exponential decay constant, k_{obsd}, is given by

$$k_{obsd} = k_{nat} + k_{nonrad} + k_{H_2O}\chi_{H_2O} \quad (1)$$

where k_{nat} is the natural rate constant for the emission of photons, k_{nonrad} is the rate constant for radiationless deexcitation processes which do not involve water, and k_{H_2O} is the rate constant for the transfer of energy to the O–H vibrations of coordinated water. For any given complex, k_{H_2O} is expected to be proportional to the number of coordinated water molecules. χ_{H_2O} is the mole fraction of water in solutions containing H_2O–D_2O mixtures. Of course, for the crystalline solids prepared from H_2O, $\chi_{H_2O} = 1$, while for substances crystallized from D_2O, $\chi_{H_2O} = 0$. In our earlier communication[16] we applied our laser excitation technique to the study of luminescence decay constants of a number of model systems (the aqua ions and various chelate complexes) and to Ln(III) ions bound to the enzyme thermolysin. By studying the various systems in both H_2O and D_2O we were able to estimate the number of water molecules coordinated to the Ln(III) ions in various circumstances. Thus, the k value measured in D_2O, $k_{obsd}^{D_2O}$, is equal to $k_{nat} + k_{nonrad}$, while $k_{obsd}^{H_2O}$ includes all three terms of eq 1. The measured difference $\Delta k_{obsd} = k_{obsd}^{H_2O} - k_{obsd}^{D_2O} = k_{H_2O}$, is the quantity of interest in that it provides a measure of the number of coordinated water molecules, q. While our initial results on solutions appeared to be quite reasonable, it was not possible to obtain the solution q values

Table II. Characterization and Luminescence Decay Constants for Ln(III) Solutions

solution	no. of water molecules in first coordination sphere		luminescence decay constant, ms^{-1}				footnotes
			Eu(III)		Tb(III)		
	Eu(III)	Tb(III)	$k_{obsd}^{H_2O}$	$k_{obsd}^{D_2O}$	$k_{obsd}^{H_2O}$	$k_{obsd}^{D_2O}$	
aquo	9.6	9.0	9.65	0.38	2.45	0.28	*a*
aquo			10.0	0.44	2.56	0.30	*b*
aquo			10.0	0.53	2.33	0.30	*c*
NTA	6.0	5.0	6.20	0.45	1.47	0.28	*d*
EDTA (1:1)	3.5	2.8	3.75	0.55	0.93	0.29	*e*
thermolysin	1.5	1.5	2.13	0.57	0.79	0.45	*f*
parvalbumin	1.2	1.3	2.02	0.78	0.76	0.45	*g*

[a] Reference 16 from the trichloride, 10^{-3} M. [b] Reference 43 from the trichloride, 0.1 M. [c] Reference 43 from the nitrate, 0.1 M. [d] Reference 16, 10^{-3} M, pH 6.0. [e] Reference 16, 10^{-3} M, pH 6.0. [f] Reference 16, $\sim 10^{-4}$ M, 0.05 M Tris buffer, pH 7.5. [g] This work, $\sim 10^{-4}$ M, 0.05 M piperazine buffer, pH 6.5.

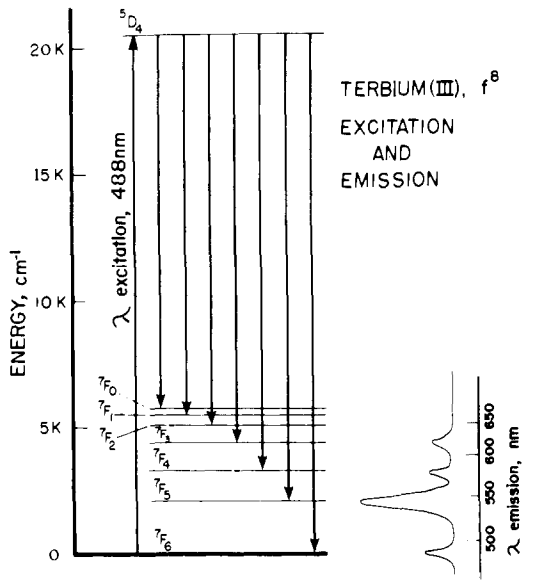

Figure 2. Electronic energy scheme for Tb(III). Radiative emission to the ground ^7F_J term is indicated by the heavy arrows with a "typical" intensity profile of the emission bands depicted to the right.

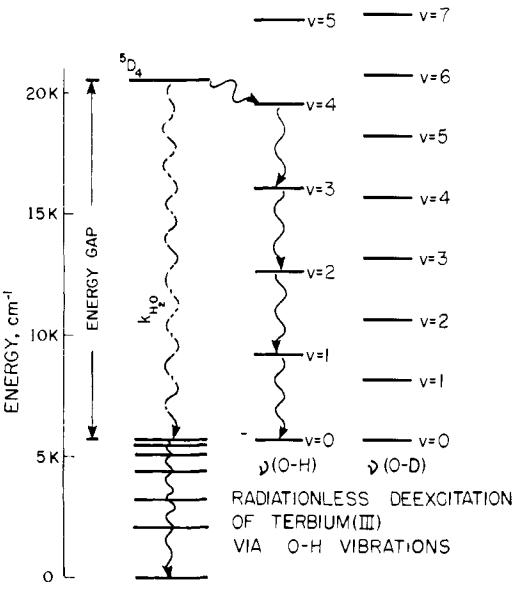

Figure 3. Radiationless deexcitation scheme for Tb(III). The significance of the energy gap between the luminescent $^5D_4$ state and the highest acceptor ($^7F_0$) state with respect to effecting nonradiative quenching of excited Tb(III) via OH oscillators is discussed in ref 46. The positions of the various vibrational overtone energies for O-H and O-D oscillators are portrayed to the right. Adapted from ref 46.

by any independent experimental means. For this reason it was deemed desirable to verify the validity of our method by an alternative experimental approach. Since the laser excitation experiments are applicable to crystalline and solution samples alike, we chose to measure the Eu(III) and Tb(III) excited-state decay constants of crystalline solids where X-ray structural determinations provide unambiguous evidence regarding the number of coordinated water molecules.

The decay constant results for the several crystalline complexes examined (Table I) reveal, as expected, that the k_{obsd} values for the H_2O species are much greater than those of the corresponding D_2O-containing systems. Our results for the ethylsulfate and chloride complexes with H_2O are in excellent accord with literature values. The systems chosen for study have numbers of coordinated water molecules varying from nine in the $[Ln(H_2O)_9]^{3+}$ ion found in the ethylsulfate and bromate salts down to zero in the dipicolinic acid complexes. In the latter complexes there are approximately 15 water molecules in the unit cell, ten of which have been crystallographically located. None of these water molecules is directly coordinated to the central lanthanide ion, although some are hydrogen bonded to oxygen atoms of coordinated carboxylate groups. Thus it is noteworthy that the measured k values are identical for crystals prepared from H_2O and from D_2O solutions. This result is supportive of our contention that only OH oscillators in the *first* coordination sphere of the metal significantly affect the luminescence decay constants.

A variety of ligand moieties occupy coordination positions in the complexes examined. These include the simple chloride and sulfate anions, carboxylate groups from a variety of ligands, chelating β-diketonate anions, amine nitrogen atoms from EDTA and NTA, and hetrocyclic nitrogen donor atoms from the nicotinate, dipicolinate, and terpyridyl ligands. The relative constancy of the $k_{obsd}^{D_2O}$ values, which are made up of k_{nat} and k_{nonrad} contributions, when compared to $k_{obsd}^{H_2O}$ suggests that most of these ligands are relatively ineffective in causing radiationless deexcitation (several of the $k_{obsd}^{D_2O}$ values found in the literature are likely too high owing to H_2O contamination; in these cases our own results are more in line with the other values, Table I). Consistent with this finding is the fact that none of the nonwater ligands has group frequencies even as high as that of the OD oscillator. The principal exception to the noted constancy in the $k_{obsd}^{D_2O}$ values occurs for the Tb(III) terpyridyl complex. In this case a ligand π^* state which lies near the $^5D_4$ emitting level may produce a large k_{nonrad} contribution. Nevertheless the Δk_{obsd} value for this system is in accord with our expectations. In the case of Eu(III) terpyridyl complex the emitting level is lower in energy and the $k_{obsd}^{D_2O}$ value is normal.

Literature results on the ethylsulfate and chloride crystals and our own on the oxalate and nitrilotriacetate (NTA) sys-

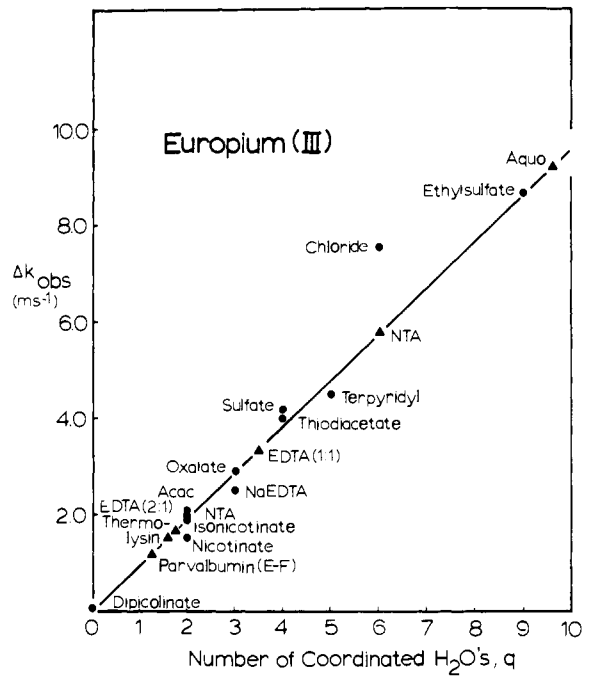

Figure 4. A plot of Δk_{obsd} vs. the number of coordinated water molecules. The solid line shown is the linear least-squares fit to the equally weighted points (chloride excluded) for the crystalline solids. The Δk_{obsd} values for the solids are indicated by closed circles ●, while those for solutions and protein-bound Eu(III) are indicated by closed triangles ▲.

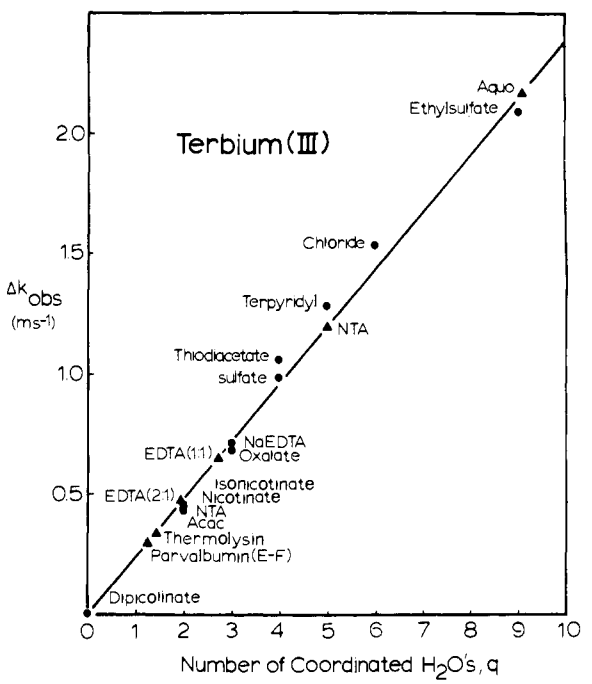

Figure 5. A plot of Δk_{obsd} vs. the number of coordinated water molecules. The solid line shown is the linear least-squares fit to the equally weighted points for the crystalline solids. The Δk_{obsd} values for the solids are indicated by closed circles ●, while those for solutions and protein-bound Tb(III) are indicated by solid triangles ▲.

tems (Table I) indicate that the k_{obsd} values are independent of the concentration of the luminescent ion in the crystal. Thus the k_{obsd} values for Eu(III) and Tb(III) ions doped into crystals containing principally Y(III) or Gd(III) are virtually identical with those of the pure concentrated crystals. Neither Y(III) nor Gd(III) has energy levels capable of accepting energy from either Eu(III) or Tb(III). Resonant energy transfer between Eu(III) ions or between Tb(III) ions very likely occurs in the concentrated solids but has no apparent influence on the measured decay constants.

One feature worthy of note is that the modest range of $k_{obsd}^{D_2O}$ values for the crystalline solids (Table I) and the protein solutions (Table II) is somewhat greater than the range observed for the model chelate systems in solution reported earlier.[16] Thus it should not be assumed that the sum of $k_{nat} + k_{nonrad}$ is constant from complex to complex. Whether the changes observed are due to changes in k_{nat} or k_{nonrad} or both is not known at present. The value of k_{nat} will be affected by the symmetry and strength of the ligand field insofar as it affects the transition probabilities. The ligand field will, of course, vary from system to system. The degree to which k_{nonrad} contributes to $k_{obsd}^{D_2O}$ is not known. Systems containing organic ligands with low-lying π-electron levels capable of accepting energy by a nonradiative mechanism have been observed to have unusually large decay constants, likely due to a sizable contribution from k_{nonrad}. It should be emphasized, however, that variations in $k_{nat} + k_{nonrad}$ from system to system do not affect the determination of the number of metal ion coordinated water molecules by the present method, since the sum of these terms is an experimentally observable quantity.

In order to assess the correlation between the luminescence decay values measured on solids and the known number of water molecules, values of Δk_{obsd} ($k_{obsd}^{H_2O} - k_{obsd}^{D_2O}$) were plotted vs. the former. The Δk_{obsd} plots for Eu(III) and Tb(III) are shown in Figures 4 and 5, respectively. The solid line shown is a linear least-squares fit to the equally weighted points for all of the crystalline solids. With the exception of the europium(III) chloride hexahydrate point, which was excluded from the least-squares analysis, the fit is quite acceptable. Deviations of the individual points from the least-squares line suggest an uncertainty of about 0.5 water molecules for measurements of this type.

Since it is our intention to establish this technique as a valid method for assessing the number of water molecules coordinated to an ion in solution, it is appropriate here to comment on the correspondence of our solution and solid-state measurements in this regard. Some controversy surrounds the problem of the number of water molecules q coordinated to lanthanide aqua ions, $Ln^{3+}aq$, in solution. A considerable body of thermodynamic and other physical evidence on solutions of Ln(III) salts reveals a discontinuous change in the measured values of most solution properties as one proceeds across the lanthanide series.[51,52] This change is such that the properties of salts of the ions La(III) through Nd(III) fall on one smoothly varying curve while those of the ions Tb(III) through Lu(III) fall on another. The properties of salts of Sm(III), Eu(III), and Gd(III) span a transitional region which connects the two above-mentioned curves in a discontinuous manner. It is not unreasonable to attribute this discontinuity to a change in q for the aqua ions from a higher to a lower number as the ionic radii decrease with increasing atomic number. Most discussions of this point suggest that this change is from q = 9 for the La–Nd series to q = 8 for the Tb–Lu sequence. On the other hand, Geier and Karlen[53] interpret their thermodynamic data so as to rule out any change in the water coordination number q of the aqua ion across the series.

On balance, we feel that the evidence for a change in q is compelling, but we suggest that this change is from q = 10 for the La–Nd series to q = 9 for the Tb–Lu aqua ions. Our reasoning and evidence for this is as follows. Stable crystalline solids (lanthanide bromates and ethylsulfates) containing the $[Ln(H_2O)_9]^{3+}$ ion are known for the entire lanthanide series including the smallest members,[26,54] e.g., $[Yb(H_2O)_9]^{3+}$. Since these materials crystallize from solutions containing the Ln(III) ions Tb(III) to Lu(III) it is unreasonable to suppose

that this nine-coordinate aqua cation is unstable in solution. The act of crystallization is likely, if anything, to induce the expulsion of water molecules from the first coordination sphere. Since $[Yb(H_2O)_9]^{3+}$ must be stable in solution, and all other evidence indicates only a single aqua ion species for the Tb(III)–Lu(III) series, it appears that this species must be $[Ln(H_2O)_9]^{3+}$. By inference then, the aqua ions of the La(III)–Nd(III) sequence are present as the ten-coordinate $[Ln(H_2O)_{10}]^{3+}$ cation. While no solid-state precedent exists for this particular ion (none exists for the $[Ln(H_2O)_8]^{3+}$ ion either!), ten-coordination is known for early members of the series; for instance, the structure of $HLaEDTA{\cdot}7H_2O$ is ten-coordinate with the singly protonated EDTA ligand occupying six coordination positions while four coordinated water molecules fill the remaining sites in the coordination sphere. The only direct evidence for eight-coordination in the Ln^{3+}aq ions comes from low-angle X-ray diffraction solution data.[55] It is not clear, however, whether experiments of this type are of sufficient accuracy to distinguish between, say, eight- and nine-coordinated water molecules. In any case these measurements were carried out on very concentrated salt solution (at or near saturation) in which a significant fraction of the total water in the solution is in the first coordination sphere of the metal ion. Under these conditions eight-coordination may well prevail. Our measurements were all carried out on dilute (≤ 1 mM) solutions.

Additional evidence supportive of the assertion that Tb^{3+}aq exists as $[Tb(H_2O)_9]^{3+}$ comes from our luminescence decay constant measurements. The Δk_{obsd} value for Tb^{3+}aq in dilute solution (2.17 ms^{-1}) corresponds almost precisely to the prediction of the least-squares line of Figure 5 for nine water molecules (2.15 ms^{-1}) and is in good agreement with the value of this parameter found for $[Tb(H_2O)_9]^{3+}$ in crystalline terbium(III) ethylsulfate nonahydrate (2.00 ms^{-1}). If our model is correct, Eu^{3+}aq should exist as an equilibrium mixture of $[Eu(H_2O)_9]^{3+}$ and $[Eu(H_2O)_{10}]^{3+}$, with perhaps an average of ~9.6 coordinated water molecules. The least-squares line through the solid-state points of Figure 4 predict a Δk_{obsd} value of 8.57 ms^{-1} for $[Eu(H_2O)_9]^{3+}$ while the observed value for Eu^{3+}aq in dilute solution is 9.27 ms^{-1}, consistent with the presence of an additional fraction of a coordinated water molecule, although this difference is scarcely outside the experimental uncertainty of our technique.

The good correspondence between our solid-state results and the solutions of the aqua ions supports the validity of our method for the estimation of q values in solution systems. Before the remaining solution results and the protein data are discussed, several interesting features of the correspondence between the solution and solid-state results should be pointed out. Firstly, the efficiency of radiationless deexcitation by a coordinated water molecule appears to be independent of the state of matter. Thus ligand exchange in solution which is rapid (10^8–10^9 s^{-1}) compared with the rates of deexcitation (10^2–10^4 s^{-1}), but is absent in the solid state, appears to have no effect on the deexcitation process. For any given ion in its excited state the probability is large that all the water molecules in the first coordination sphere will have exchanged with bulk solvent water many times before deexcitation occurs. Clearly if there is a rapid equilibrium between species differing in q, the solution luminescence decay rate results will correspond to a time-averaged value. Another conclusion which may be drawn from the close correspondence between the solution and solid-state results in that the radiationless deexcitation process is affected only by the constitution of the first coordination sphere. Various phonon processes, next nearest neighbor effects, and the influences of resonant energy transfer appear to be absent or negligible.

A few of our solution results have been placed on the least-squares line of the plots of Δk_{obsd} vs. q obtained for the crystalline solids (Figures 4 and 5). The value of q corresponding to these points represents a prediction of the average number of coordinated water molecules for a particular solution species. These values have been collected in Table II. For systems involving 1 equiv of the tetradentate tripod-like nitrilotriacetate (NTA) ligand the Eu(III) and Tb(III) complexes have predicted q values of 6.0 and 5.0, respectively. This result is consistent with nine-coordination in the case of Tb(III) and a ten-coordinate species in the case of Eu(III). For solutions containing 1 equiv of the sexadentate EDTA ligand, the Eu(III) and Tb(III) q values are 3.5 and 2.8, respectively. This again, within the probable uncertainty in q of ± 0.5, is in good accord with the above model. Indeed there is spectroscopic evidence in the case of the Eu(III) EDTA system[56-59] that an equilibrium exists between two species differing, it is presumed, by one molecule of coordinated water. Our results are consistent with the following equilibrium:

$$Eu^{III}EDTA(H_2O)_3 + H_2O \leftrightarrows Eu^{III}EDTA(H_2O)_4 \quad (2)$$

with an equilibrium constant on the order of unity. This finding is consistent with solution thermodynamic data.[59]

Since our method is intended to characterize lanthanide binding sites in biological macromolecules, we have examined two such molecules in which the Ln(III) ion binding has been studied by X-ray crystallography. X-ray studies on the endoproteinase thermolysin reveal that in the native state it binds a Zn(II) ion at the active site as well as four Ca(II) ions which appear to play a structural role. Two of the Ca(II) ions occupy a double site (sites 1 and 2) only 3.8 Å apart, while the other two are bound individually at well-separated locations (sites 3 and 4). It has been shown that a Ln(III) ion will readily replace the Ca(II) ion at site 1 with the concomitant expulsion of its double site Ca(II) partner. Under conditions of high Ca(II) concentration (1 mM) in the buffer, the Ca(II) ions at sites 3 and 4 are not replaced by Ln(III) ions. Our luminescence decay constant measurements on Eu(III) and Tb(III) bound at Ca(II) site 1 in thermolysin (Table II) suggest that about 1.5 water molecules are bound to the Ln(III) ion under these conditions. This is within experimental uncertainty of either one or two water molecules. In the native state the Ca(II) ion at site 1 is coordinated by a single water molecule, but in addition is bridged to its partner Ca(II) at site 2 by three carboxylate groups. When the partner Ca(II) ion is expelled upon Ln(III) binding this bridging structure is necessarily disrupted. It is unlikely that the X-ray electron density maps of this region are of sufficient accuracy to tell whether or not a second water molecule binds to the Ln(III) ion at site 1 as a consequence of this structural rearrangement. Our results are clearly consistent with the structural information available.

The second protein that we examined is a muscular parvalbumin isolated from the common mirror carp, which binds two Ca(II) ions in the native state. The structure of one of the isotypes has been determined and the binding of Ln(III) ions at the EF Ca(II) site has been characterized by X-ray crystallography.[6] The Ca(II) ion at the solvent-accessible EF site is known to bind a single water molecule. Our results (Table II) are consistent with this finding. The good correspondence between the results of our luminescence experiments on these two proteins and the expectations based on independent structural information demonstrates the utility of our method in the study of coordination of macromolecule-bound Ln(III) ions.

Conclusions

The linear relationship between the $(k_{obsd}{}^{H_2O} - k_{obsd}{}^{D_2O})$ values and the number of water molecules coordinated to Ln(III) ions has been verified by measurements on a series of structurally well-characterized crystalline complexes of

Eu(III) and Tb(III). The good correspondence between solution and solid-state results implies that the nonradiative deexcitation process via coordinated H_2O is independent of the state of matter. This finding suggests that the method may be useful in the study of heterogeneous systems (e.g., membrane bound ions) and for solid state–solution state comparisons.

Our results on two structurally well-characterized proteins demonstrates the feasibility of using the present extremely simple technique in the study of biological molecules. The only alternative experimental means by which to obtain information regarding the number of coordinated water molecules, q, is by the use of bound Gd(III) as a water proton (or deuteron) resonance relaxation probe.[60-62] This requires NMR measurements at several spectrometer frequencies and the fitting of the results to a multiparameter equation. It may be that the present much simpler experimental method may be useful when used in conjunction with resonance relaxation measurements in order to allow the remaining parameters to be determined with greater accuracy.

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