Laser-Induced Luminescence Studies of $Eu(HAM)^{3+}$. An Evaluation of Complex Stability and the Use of the Gadolinium Analog as a Relaxation Agent in ^{89}Y NMR

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The hexaazamacrocyclic (HAM) complex $Eu(C_{22}H_{26}N_6)^{3+}$ has been characterized via laser-induced Eu^{3+} luminescence in an effort to identify the potential biological probe function of the Eu^{3+} and Gd^{3+} macrocyclic species. Examination of the $Eu(HAM)^{3+}$ $^7F_0 \rightarrow {}^5D_0$ excitation spectra (578–581 nm) and excited state lifetime values as a function of pH reveals a species conversion which occurs on the timescale of the luminescence lifetime (0.063 ms). The sensitivity of the Eu^{3+} luminescence technique is exploited in a thorough evaluation of the stability of the $Eu(HAM)^{3+}$ complex. After 48 h in solution at pH 7.0 and 25 °C, approximately 4% of the macrocycle decomposes. The presence of an added excess of the strong chelating agent diethylenetriaminepentaacetic acid (dtpa) or a competing metal ion (Y^{3+}) does not increase the rate of decomposition. However, strong pH and temperature dependencies are observed for the rate of $Eu(HAM)^{3+}$ degradation, with the elevation of the temperature to 37 °C causing the largest increase in the decomposition rate (12% in 48 h). The $Gd(HAM)^{3+}$ complex is an effective ^1H NMR relaxation agent that has been proposed for use in magnetic resonance imaging (MRI) (Smith, P. H.; Brainard, J. R.; Morris, D. E.; Jarvinen, G. D.; Ryan, R. R. *J. Am. Chem. Soc.* **1989**, *111*, 7437–7443). Currently, $Gd(dtpa)^{2-}$ is the most commonly used contrast agent for MRI. In a comparison of the ability of $Gd(HAM)^{3+}$ and $Gd(dtpa)^{2-}$ to relax the ^{89}Y NMR nucleus, the concentration of $Gd(HAM)^{3+}$ required to achieve maximum signal enhancement of the single ^{89}Y NMR resonance of $Y(dtpa)^{2-}$ is one-tenth of that necessary for $Gd(dtpa)^{2-}$.

Introduction

Nature's use of macrocyclic ligands for metal ions in biological systems (e.g. porphyrins) has provoked interest in modelling these cyclic structures. A recent report[1] of the synthesis of a lanthanide HexaAzaMacrocycle (HAM, subject of the present study), $Ln(C_{22}H_{26}N_6)(CH_3COO)_2Cl{\cdot}nH_2O$ (n = 1–4) for Ln = La→Lu, except radioactive Pm, describes a class of molecules which are unusually heat stable (decomposition occurs at 240 °C) and inert in the presence of typical lanthanide ion precipitating agents (e.g. F^-, OH^- $C_2O_4^{2-}$). It appears that the unique properties of these complexes arise from a match between the size of the cation and the macrocyclic cavity, and the unavailability of an "unwrapping" mechanism for metal ion release.

The demonstrated thermodynamic stability and kinetic inertness of $Ln(HAM)^{3+}$ has led to the investigation of possible uses for this lanthanide macrocycle. The ability of certain lanthanide ions to luminesce at room temperature, and their capacity to act as nmr shift and/or relaxation reagents, renders $Ln(HAM)^{3+}$ complexes potential probes for some of the same biological systems they were initially intended to model. For example, Smith et al.[2] proposed the use of the Gd^{3+} analog as a contrast agent for magnetic resonance imaging (MRI), based on its kinetic inertness and ability to relax water protons more effectively than Gd^{3+}_{aq} at 7.0 T and 25 °C. A recent report by Morrow and coworkers indicates that the La-, Eu-, Gd-, and $Tb(HAM)^{3+}$ complexes promote the cleavage of RNA via the transesterification of the phosphodiester linkage of RNA;[3] in particular $Eu(HAM)^{3+}$ displays catalytic behavior. Both of these applications require that the lanthanide ion remain complexed by the macrocycle, as the free metal ion and/or the free ligand are toxic, and therefore unacceptable for in vivo applications.

The reported[2] relaxivity and stability of $Ln(HAM)^{3+}$ prompted us to investigate the Gd^{3+} complex for use as a relaxation agent in our ^{89}Y nmr studies of calcium-binding proteins. Previous studies of Y^{3+}-bound pike parvalbumin[4] successfully employed the relaxation agent $Gd(dtpa)^{2-}$ (dtpa = diethylenetriaminepentaacetic acid); however, we are interested in a more effective and more kinetically inert relaxation complex. It is essential that the metal ion remain encapsulated by the macrocycle to prohibit exchange between protein-bound Y^{3+} ions and Gd^{3+} ions added as the relaxation agent.

Laser-induced Eu^{3+} luminescence is a well-established tool for the characterization of Eu^{3+} complexes.[5] By exciting the Eu^{3+} ion from its $^7F_0$ ground state to the $^5D_0$ excited state and monitoring the emission to the $^7F_2$ level, the number of individual Eu^{3+} environments, speciation, complex stoichiometry, and the number of water molecules bound in the first coordination sphere can be obtained.[5] The technique is capable of detecting luminescence from nanomolar concentrations of Eu^{3+} complexes. In the present research, the sensitivity of laser-induced Eu^{3+} luminescence has been exploited in a rigorous evaluation of the stability of $Eu(HAM)^{3+}$. Gd^{3+} and its periodic table neighbor, Eu^{3+}, possess similar ionic radii (0.938 Å vs 0.950 Å), and binding characteristics; therefore, studies of $Eu(HAM)^{3+}$ can be directly applied to the Gd^{3+} analog. In addition, we report the results of experiments using $Gd(HAM)^{3+}$ as a relaxation agent in ^{89}Y nmr studies. $Gd(HAM)^{3+}$ and $Gd(dtpa)^{2-}$ are compared with respect to their ability to enhance the ^{89}Y nmr spectrum of $Y(dtpa)^{2-}$, a polyaminocarboxylic acid model of metal binding sites in calcium-binding proteins.

Experimental Section

Synthesis of $M(HAM)^{3+}$ Complexes. Synthesis of $M(HAM)(CH_3COO)_2Cl{\cdot}3H_2O$ ($M = Eu^{3+}, Gd^{3+}$) was accomplished according to the procedure outlined by De Cola et al.[1] $Eu(CH_3COO)_3$, $Gd(CH_3COO)_3$, 2,6-diacetylpyridine, and ethylenediamine were obtained from Aldrich

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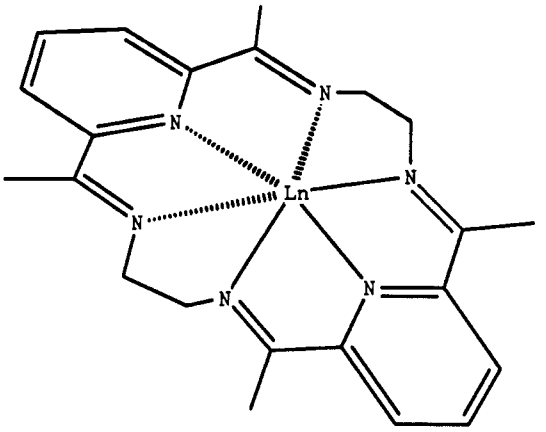

Figure 1. Structure of Ln(HAM)$^{3+}$ (Ln = Gd^{3+}, Eu^{3+}) in this account.

Chemical Co. All other chemicals were reagent grade. The ^{1}H NMR spectrum of Eu(HAM)$^{3+}$ in both D_2O and $CDCl_3$ was recorded at 25 °C on a Bruker AM-300 spectrometer. Elemental analysis of the Gd^{3+} analog was performed by Galbraith Laboratories (Knoxville, TN) and was consistent with the formula $GdC_{26}H_{32}N_6O_4Cl\cdot 3H_2O$.

Laser-Induced Eu^{3+} Luminescence Measurements. A Nd:YAG (Continuum Series YG518C) pumped tunable dye laser (Model TD150) pulsed at 10 Hz was used to obtain the excitation spectra and lifetime data. The details of this system are presented elsewhere.[6] A mixture of Rhodamine 590 (Exciton) and Rhodamine 610 (Kodak) dyes was used to excite the $^7F_0 \rightarrow {}^5D_0$ transition of the Eu^{3+} ion (578–581 nm), while monitoring the $^5D_0 \rightarrow {}^7F_2$ emission at 614 nm. Component peaks of the excitation spectra were deconvoluted using the program SPECTRUM,[7] while luminescence decays were analyzed into a sum of component exponential functions using the program LIFETIME.[8] Both programs were developed in this laboratory for the IBM CS9000 computer. In addition, the commercially available program PeakFit (Jandel Scientific) was employed in the data analysis. All of the programs employ algorithms based on the nonlinear regression method developed by Marquardt.[9] The quality of the fit was judged by the minimization of χ^2 and by plots of the weighted residuals of a fit of the data; symmetric deviations from zero were taken as evidence of a good fit.

pH and Stability Studies of Eu(HAM)$^{3+}$. The excitation spectra and excited state lifetime values of 0.2 mM Eu(HAM)$^{3+}$ samples were recorded over the pH range 5.0–10.0. Each sample was buffered by using a combination of 0.025 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES) and 0.025 M piperazine (Sigma Chemical Co.) in the presence of 0.5 M KCl. All $^7F_0 \rightarrow {}^5D_0$ spectra were recorded over the 578–581-nm range and curve-resolved into the individual excitation bands using nonlinear regression methods.

For the stability studies, a series of solutions containing 0.2 mM Eu(HAM)$^{3+}$ were prepared over the course of ~50 h and thermostated at either 25 ± 0.5 or 37 ± 0.5 °C through the use of a Thermolyne Dri-Bath (Sybron Corp.). The solutions were buffered using 0.05 M HEPES (pH 7.0, 7.4) or 0.05 M piperazine (pH 5.8) with either 0.15 or 0.5 M KCl. pH and KCl concentrations employed were chosen to match experimental conditions required for the intended applications of Gd(HAM)$^{3+}$. At the end of known periods of incubation of Eu(HAM)$^{3+}$ in solution, any uncomplexed Eu^{3+} was sequestered by 0.8 mM dtpa which was added immediately prior to the luminescence measurement. From the Eu(dtpa)$^{2-}$ excitation peak intensity, the concentration of Eu(dtpa)$^{2-}$ present in each sample was interpolated from a standard curve; this value was used to calculate the amount of Eu(HAM)$^{3+}$ that had decomposed.

^{89}Y Nuclear Magnetic Resonance Studies. All ^{89}Y NMR spectra were recorded at 25 °C on a Bruker AM-300 Fourier transform spectrometer at 7.05 T where ^{89}Y resonates at 14.75 MHz. A 21° pulse (7.0 μs) with a 4.0-s relaxation delay was typically used. The acquisition time for each pulse was 1.606 s, and the resulting free induction decay contained 32K data points. Each spectrum comprises 160 pulses which were recorded over approximately 15 min. Y(dtpa)$^{2-}$ samples (0.3 M) were prepared in 0.05 M piperazine buffer at pD 6.0. The solutions were ~70% in D_2O to provide a lock signal. Improvement in the signal-to-noise ratio (S/N) of the Y(dtpa)$^{2-}$ peak at 82.6 ppm (relative to 1.0 M YCl_3 at 0.0 ppm) was monitored as a function of the concentration of Gd(HAM)$^{3+}$ or Gd(dtpa)$^{2-}$ added as the relaxation agent. All metal ion solutions were standardized by a chelometric technique.[10]

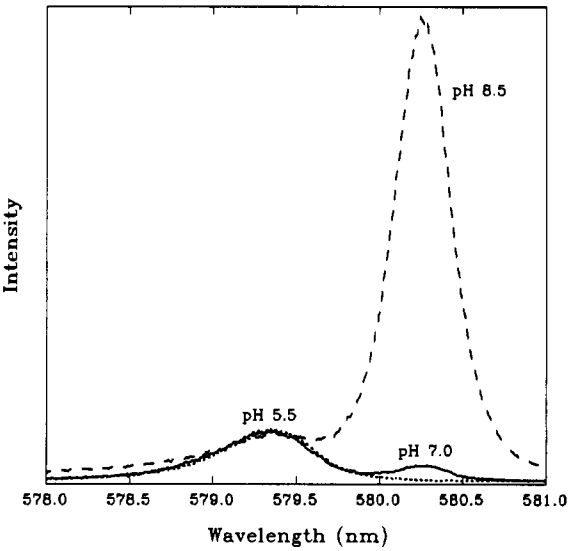

Figure 2. $^7F_0 \rightarrow {}^5D_0$ excitation spectrum (λ_{em} = 614 nm) of 0.2 mM Eu(HAM)$^{3+}$ at pH 5.5 (···), 7.0 (—), and 8.5 (- - -).

Results and Discussion

Laser-Induced Eu^{3+} Luminescence Spectroscopy. Previous work conducted in this laboratory has examined the luminescence properties of the Eu^{3+} ion incorporated into a polyamino polycarboxylate macrocycle (DOTA),[11] carboxymethyl–macrocyclic ether–bis(lactones),[12] and macrocyclic diaza crown ether complexes containing carboxylate ligating groups.[13] Although the nitrogen atom is a strong ligand for Ln^{3+} ions in the presence of noncoordinating solvents,[14] complexes with pure nitrogen donors typically do not exist in aqueous solution due to competition from water molecules or hydroxide ions. The present spectroscopic investigation of Eu(HAM)$^{3+}$ is unique in its characterization of the Eu^{3+} ion encapsulated by a macrocycle containing solely nitrogen atoms. In addition, our study permits a detailed evaluation of the stability of Eu(HAM)$^{3+}$ in solution.

The ground ($^7F_0$) and emissive ($^5D_0$) excited states of the Eu^{3+} ion are nondegenerate. Since neither level is split by the ligand field, in principle, each Eu^{3+} ion environment gives rise to a distinct band in the excitation spectrum. However, the $^7F_0 \rightarrow {}^5D_0$ electronic transition is Laporte forbidden, resulting in a low molar absorptivity ($\epsilon \sim 10^{-2}$ M^{-1} cm^{-1}). The symmetry of the Eu(HAM)$^{3+}$ molecule in solution (D_{2h})[2] also contributes to the low intensity $^7F_0 \rightarrow {}^5D_0$ excitation band observed for Eu(HAM)$^{3+}$. In our experiments, it was necessary to use sample concentrations of 0.2 mM (20–200 times higher than usual) in order to achieve an acceptable signal-to-noise ratio. The excitation band (579.38 nm) obtained for Eu(HAM)$^{3+}$ at pH 5.5 (Figure 2) is consistent with a single species present in solution. However, the shape and intensity of the excitation spectrum change drastically as a function of pH. At pH values greater than 6.5, a second peak appears at 580.26 nm (Figure 2). The emission intensity of this band varies

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with pH such that a 10-fold intensity increase is observed between pH 6.5–8.5. From the pH induced spectral changes we estimate a midpoint pH of ~8.2 for the species conversion.

The X-ray crystal structure of $Gd(HAM)^{3+}$ shows that the six N atoms in the macrocycle and two bidentate acetate anions are coordinated to the metal ion.[2] In solution, the carboxylate oxygen atoms of the acetate ion are available for ligation to the Eu^{3+} ion at pH 5.5 ($pK_a(CH_3COOH)$ = 4.8); however, upon addition of excess acetate (5–100 mM) to the $Eu(HAM)^{3+}$ sample, no increase in intensity was observed for either the 579.38-nm or the 580.26-nm excitation bands. Instead, a new peak appeared in the excitation spectrum at 579.85 nm (data not shown) presumably due to $Eu(HAM)^{3+}$ with coordinated acetate anions. This implies that under the experimental conditions employed the acetate ion does not remain coordinated to the Eu^{3+} ion in solution. This is not surprising since H_2O is a particularly strong ligand toward Ln^{3+} ions, and would be expected to compete effectively for the remaining coordination sites. In neutral or acidic solutions competition from H_2O prevents coordination by any but the strongest donor ligands.

According to the relationship established by Albin and Horrocks,[15] an increase in the number of negatively charged groups surrounding the Eu^{3+} ion results in a red shift of the observed excitation maximum. As the pH of the $Eu(HAM)^{3+}$ sample is increased, the replacement of bound H_2O molecules by OH^- could give rise to the excitation band at 580.26 nm. An $Eu(HAM)^{3+}$ species with a mixture of coordinated H_2O and OH^- ligands would also have lower symmetry in solution, rendering the $^7F_0 \rightarrow {}^5D_0$ transition more allowed and thus increasing the molar absorptivity. In addition, since the $^5D_0$ excited state of the Eu^{3+} ion is deexcited by a mechanism which involves the OH oscillators (H_2O or OH^-) in the first coordination sphere, a reduction in the number of OH oscillators coordinated to the Eu^{3+} ion would also result in a luminescence intensity increase.

The number of coordinated water molecules, q, may be calculated according to eq 1,[5] where τ^{-1} is the reciprocal of the

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

excited state lifetime (ms^{-1}) in the solvent indicated. For the $Eu(HAM)^{3+}$ system, determination of the number of H_2O molecules is complicated by the unexpected observation of a "humped" luminescence decay curve for excitation at 579.3 nm, and a decay curve which falls off rapidly at first, followed by a much slower decay for excitation at 580.3 nm (Figure 3). This nonexponential behavior indicates that the species giving rise to the peaks at 579.38 and 580.26 nm are in *intermediate exchange*.[16] In this process, the rate of chemical interchange between two species is comparable to the rate of luminescence decay. In contrast, two species, A and B, in equilibrium in solution are in *rapid exchange* if the rate of interconversion is much greater than the characteristic deexcitation rate for each species. In the latter case two peaks are observed in the excitation spectrum, but only a single excited state lifetime value is resolved with an exponential decay that is representative of a weighted average of the two species A and B. Alternatively, two species present in equilibrium in solution exhibit *slow exchange* if the rate of interconversion between species A and B is much slower than the deexcitation rate. Each complex then exhibits a separate peak in the excitation spectrum with an excited state lifetime of its own, such that selective excitation of either species A or B yields an exponential decay with a characteristic lifetime, τ_A or τ_B.

Intermediate exchange behavior has been observed by Horrocks and co-workers[16] in a ternary Eu^{3+} complex of iminodiacetic acid (imda) and *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (dcta), $Eu(dcta)(imda)^{3-}$. Through judicious choice of excitation and emission wavelengths, they were able to identify the luminescent species. However, unlike the $Eu(dcta)(imda)^{3-}$ case, it is not possible to separate spectroscopically the luminescent entities responsible for this phenomenon in $Eu(HAM)^{3+}$ as no emission wavelength exists where one species is dominant. The "humped" decay pattern produced by exciting $Eu(HAM)^{3+}$ at 579.3 nm reflects the conversion of a species with a lower intrinsic luminescence to one with a higher intrinsic luminescence. The emission intensity maximum is not achieved until a good portion of species A has converted to species B. In contrast, selective excitation with laser light pulsed at 580.3 nm produces a decay curve which reflects the conversion of a species with high intrinsic luminescence to one of lower fractional luminescence. The analysis of excited state lifetime data displaying intermediate exchange behavior is more complex than either the rapid exchange or the slow exchange case, and incorporates both the interconversion rate constants (k_A, k_B) and the deexcitation rate for species A and B (τ_A^{-1}, τ_B^{-1}).

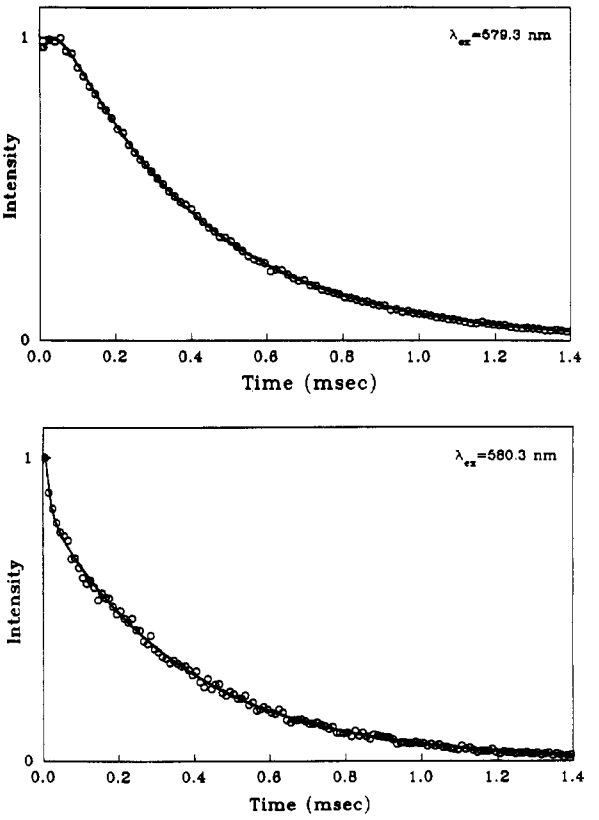

Figure 3. Luminescence decay curves for excitation of a 0.2 mM $Eu(HAM)^{3+}$ solution (pH 8.3). The solid lines are the theoretical fit of the data using the intermediate exchange model described in the text. The excitation wavelength employed is indicated to the right of each curve.

The following equations describe the processes that can occur as a result of selective excitation of species A to form excited A*:

$$A^* \xrightarrow{\tau_A^{-1}} A + h\nu + \text{nonradiative loss} \quad (2)$$

$$A^* \xrightarrow{k_A} B^* \quad (3)$$

$$B^* \xrightarrow{\tau_B^{-1}} B + h\nu + \text{nonradiative loss} \quad (4)$$

$$B^* \xrightarrow{k_B} A^* \quad (5)$$

Equations 2 and 4 represent the radiative and nonradiative

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Table I. Data for Determination of the Number of Water Molecules, q, Coordinated to Eu(HAM)$^{3+}$ as a Function of pH

pH	H_2O		D_2O	q^a	no. of OH oscillators
	τ (ms)	τ^{-1} (ms^{-1})	τ^{-1} (ms^{-1})		
5.77	0.28	3.6	0.48	3.3 ± 0.5	6–7
6.41	0.28	3.6	0.48	3.3 ± 0.5	6–7
6.94	0.32	3.1	0.43	2.8 ± 0.5	5–6
7.47	0.36	2.8	0.43	2.5 ± 0.5	5
8.01	0.45	2.2	0.45	1.8 ± 0.5	3–4
8.44	0.54	1.9	0.50	1.5 ± 0.5	3

[a] Reported uncertainty stems from the empirical fit of the data used to determine eq 1 (Horrocks and Sudnick, 1981).

deexcitation of A* and B*, respectively, while eqs 3 and 5 depict the chemical interconversion of A* into B* and vice versa. The differential equations describing these processes are given in eqs 6 and 7; their solutions have been reported previously.[16]

$$\frac{d[A^*]}{dt} = k_B[B^*] - (k_A + \tau_A^{-1})[A^*] \quad (6)$$

$$\frac{d[B^*]}{dt} = k_A[A^*] - (k_B + \tau_B^{-1})[B^*] \quad (7)$$

At acidic pH values where only a single peak is observed in the Eu(HAM)$^{3+}$ excitation spectrum, a single exponential with τ = 0.28 ms (τ^{-1} = 3.6 ms^{-1}) fits the excited state decay. Application of eq 1 indicates that three H_2O molecules (six OH oscillators) are coordinated to Eu(HAM)$^{3+}$ at low pH, in good agreement with previous reports.[2] Using the intermediate exchange model to analyze the observed excited state lifetime decays, we find values for τ_B which increase with increasing pH (Table I); τ_A was held constant at 0.28 ms (τ_A^{-1} = 3.6 ms^{-1}), the value determined in the absence of intermediate exchange. The best fit for the forward pseudo-first-order rate constant (k_A) of the chemical exchange is 16 ± 1 ms^{-1}. The reported uncertainty is the root mean square deviation in values obtained in the pH range 7.4–8.4, where the data exhibits the most pronounced intermediate exchange behavior. At pH values above and below this range intermediate exchange behavior is not as well defined. For the same pH range, acceptable fits to the data were obtained using k_B values in the range 20–40 ms^{-1}.

In support of our hypothesis, the increase in the excited state lifetime value as a function of pH indicates a loss of OH oscillators surrounding the Eu^{3+} ion. Based on the reciprocal of the excited state lifetime values in H_2O (1.9 ms^{-1}) and D_2O (0.50 ms^{-1}) at pH 8.4, we calculate 1.5 water molecules (three OH oscillators from H_2O or OH$^-$) bound to the Eu^{3+} ion; a decrease of half the number present at pH 5.8–6.4 (Table I). We propose that the band which arises at 580.26 nm is due to Eu(HAM)$^{3+}$ with a mixture of OH$^-$ and H_2O ligands (i.e. Eu(HAM)$(OH)_x(H_2O)_y$). Furthermore, the stepwise removal of protons reduces the nonradiative deexcitation rate of the $^5D_0$ excited state, thereby creating a more luminescent molecule. Holz et al.[13] have observed a red-shifted species in the excitation spectrum and a concurrent lengthening of the excited state lifetime consistent with monodeprotonation of the two H_2O molecules coordinated to the Eu^{3+} complex of the macrocyclic ligand K21DA. A similar trend also has been reported for the Eu^{3+}_{aq} ion;[17] the intensity of the excitation maximum increases 70-fold over the pH range 5–7.

While the deprotonation of bound water molecules can satisfactorily explain changes in the shape and intensity of the Eu(HAM)$^{3+}$ excitation spectrum, it most likely is not the source of the observed intermediate exchange behavior, since proton loss by a water molecule is expected to be a rapid event. To explain the exchange phenomenon, we suggest that a pH dependent conformational change occurs. The "hinge" observed in the X-ray crystal structure of Gd(HAM)$^{3+}$, and the flexibility of the macrocycle in solution as verified by the degeneracies in the ^1H and ^{13}C NMR spectra,[2] lend credence to the idea that a change in the conformation of the macrocycle may be involved. Alternatively, a bimolecular process may occur, such as the formation of a μ-hydroxo bridged dimeric or polymeric species. While polymerization of lanthanide complexes of the polyamino carboxylic acids hedta (*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid) and edta occurs above pH 9,[18] Brittain[19] has observed the self-association of complexes of Eu^{3+} or Tb^{3+} with aspartic acid between pH 6.0 and pH 10. Results of potentiometric titrations[20] suggest that the oligomers could be caused by a bridging hydroxide ligand. For the Eu(HAM)$^{3+}$ system it is possible that sample concentration plays a role in the formation of a polynuclear species as experiments conducted at lower Eu(HAM)$^{3+}$ concentrations (100 μM → 5 μM) showed a progressive loss of the intermediate exchange behavior with decreasing concentration. However, no change in the relative amplitudes of the two bands in the excitation spectrum was observed as a function of concentration.

Eu(HAM)$^{3+}$ Stability in Aqueous Solution. The lanthanide complexes of crown ether analogs of HAM decompose readily in aqueous solution.[21] However, in an ^1H NMR investigation of Gd(HAM)$^{3+}$ for potential use as a magnetic resonance imaging agent, Smith and co-workers reported that only 2.2% of the Eu(HAM)$^{3+}$ complex was lost in 6 days (144 h) at pH 6.6 and 25 °C.[2] Owing to the sensitivity of our laser-induced luminescence technique, we felt it worthwhile to reinvestigate the stability of Eu(HAM)$^{3+}$ in aqueous solution. Since luminescence intensity is directly proportional to species concentration, we initially attempted to detect complex loss based on a decrease in the intensity of the Eu(HAM)$^{3+}$ excitation maxima. However, it was difficult to quantitate accurately minor changes in the intensity of the Eu(HAM)$^{3+}$ excitation spectrum, which is comprised of fairly broad bands (fwhm = 25 and 13 cm^{-1} for the 579.38- and 580.26-nm peaks, respectively). Therefore, we chose to monitor directly the amount of Eu^{3+} released upon decomposition of the Eu(HAM)$^{3+}$ complex as a function of time. In order to detect the uncomplexed Eu^{3+}, we chose to sequester it using the chelating ligand dtpa based on three criteria: (1) Eu(dtpa)$^{2-}$ is a highly luminescent species, which yields (2) a single excitation band at 579.86 nm, a wavelength which does not coincide with the excitation maxima observed for Eu(HAM)$^{3+}$ (Figure 4); and (3) the high formation constant (K_f^{Eu} = $10^{22.9}$)[22] guarantees that any uncomplexed Eu^{3+} will be bound by the dtpa. From a standard curve (Figure 5, inset) generated by using Eu(dtpa)$^{2-}$ samples of known concentration, we interpolated the Eu(dtpa)$^{2-}$ concentration present in each incubated Eu(HAM)$^{3+}$ sample and, consequently, the amount of Eu^{3+} released from the macrocycle. At pH 7.0, 0.5 M KCl, and 25 °C, we find that ~4% of the Eu(HAM)$^{3+}$ complex decomposed in 48 h (Figure 5, open circles). This represents a decomposition rate 5.5 times that observed by Smith and co-workers.[2]

Prior investigations[2,3] of Eu(HAM)$^{3+}$ stability noted a significantly higher amount of Eu^{3+} ion removal from the macrocycle when the samples were incubated in the presence of dtpa. In a separate experiment, we incubated the Eu(HAM)$^{3+}$ with a 4-fold excess of dtpa instead of adding the chelating agent immediately prior to recording the excitation spectrum. No difference was observed; under these conditions the amount of Eu^{3+} liberated

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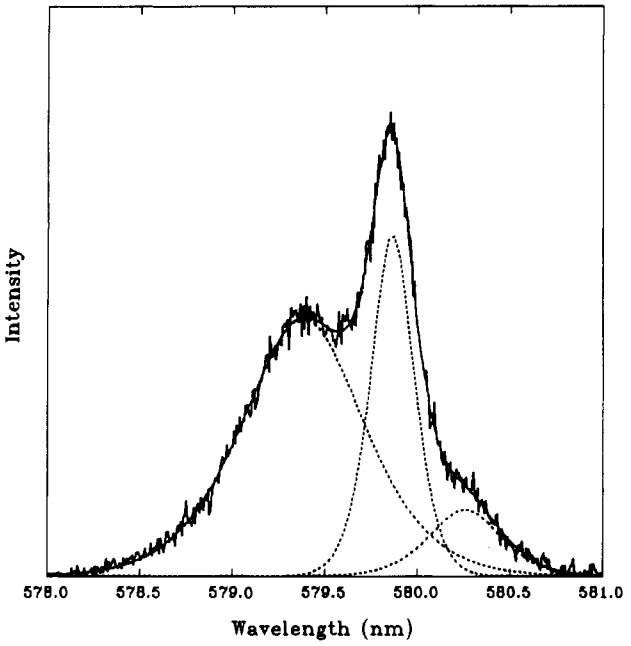

Figure 4. Curve-resolved $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of 0.2 mM $Eu(HAM)^{3+}$ (pH 7.0) in the presence of the chelating agent dtpa (0.8 mM). Component peaks were deconvoluted using a Lorentzian–Gaussian lineshape to fit the data. The parameters of the fit are as follows: λ_1 = 579.38 nm, σ_1 = 0.77 nm; λ_2 = 579.86 nm, σ_2 = 0.27 nm; λ_3 = 580.26 nm, σ_3 = 0.45 nm (λ is the wavelength of the peak maximum, and σ is the full width at half-maximum).

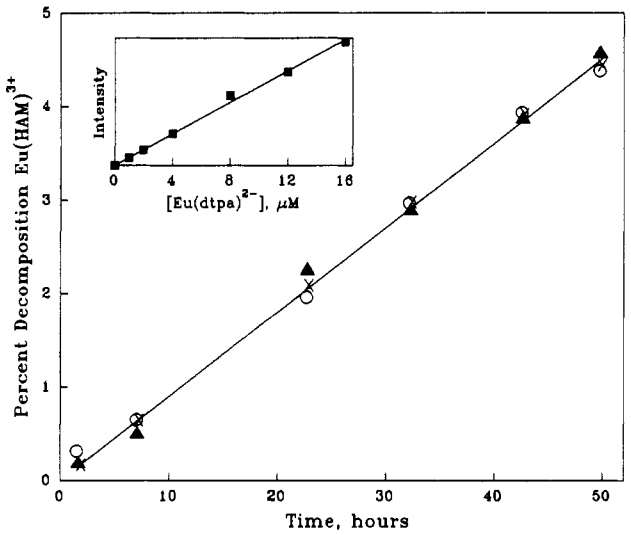

Figure 5. Comparison of the stabilities of (○) 0.2 mM $Eu(HAM)^{3+}$ (pH 7.0, 0.5 M KCl, 25 °C) and samples incubated in the presence of (▲) 0.8 mM dtpa and (×) 0.8 mM Y^{3+}. Inset graph: Standard curve used in the stability studies. Intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation band (579.86 nm) of $Eu(dtpa)^{2-}$ is plotted for samples of known concentration.

from the macrocycle is also 4% in 48 h (Figure 5, triangles). It appears from these results that the complex degrades in a dissociative manner as opposed to a mechanism which involves the extraction of the metal ion from the macrocycle with the aid of an external ligand. The inability of dtpa to remove the Eu^{3+} ion from the macrocycle implies that the protein metal-binding sites (which polyamino carboxylate ligands are often used to model) will not compete with the macrocycle for the complexed lanthanide ion. This property of the $Ln(HAM)^{3+}$ complex is particularly important for our intended use of the Gd^{3+} analog as a relaxation agent in ^{89}Y NMR studies of Ca^{2+}-binding proteins owing to the length of time required to collect the NMR data (24–48 h).

Analogs of HAM in which the pyridine nitrogen atoms have been replaced by oxygen atoms are known to undergo transmetalation reactions.[23] Indeed, this behavior is useful synthetically in cases where the metal ion of interest is incapable of templating the macrocycle formation (e.g. transition metals); however, it is detrimental if loss of the metal ion from the complex disrupts its intended probe function or is toxic to the system under study. The ability of lanthanide ions to mediate metal ion exchange in acyclic complexes has been demonstrated by Breen and co-workers[24] in a study of the ligand exchange kinetics between Tb^{3+} and $Ca(edta)^{2-}$. Therefore, it was considered possible that $Ln(HAM)^{3+}$ was susceptible to a similar effect. To evaluate the stability of $Eu(HAM)^{3+}$ in the presence of another metal ion, we incubated $Eu(HAM)^{3+}$ with a 4-fold excess of Y^{3+} and monitored the release of Eu^{3+} from the macrocycle (enough dtpa was added immediately prior to the luminescence measurement to bind all of the free Y^{3+} and Eu^{3+}). The lanthanide-like Y^{3+} ion was chosen for this experiment because it does not interfere with the Eu^{3+} luminescence measurements and the fact that it is the potential source of competition with the Gd^{3+} in our NMR experiments. In 48 h ~4% of the starting sample decomposed, a result identical to that obtained in the presence and absence of dtpa (Figure 5).

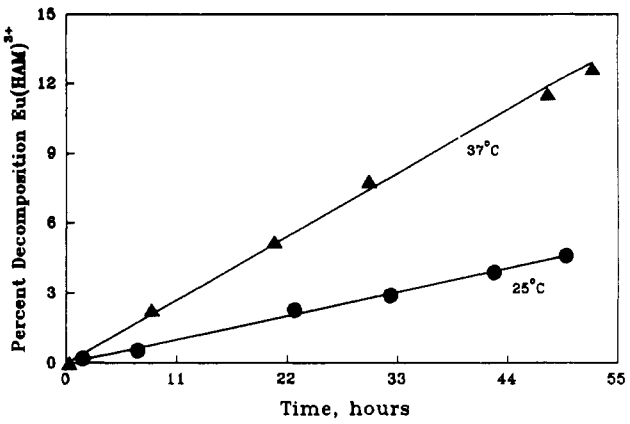

Figure 6. Comparison of the degradation of 0.2 mM $Eu(HAM)^{3+}$ samples (pH 7.0, 0.5 M KCl) incubated at (●) 25 °C and (▲) 37 °C.

The insensitivity of the HAM molecule to the presence of a strong chelating agent or a competing metal ion prompted us to examine other experimental variables, namely pH, temperature, and salt concentration. All of the experiments described above were conducted at pH 7.0, 25 °C, with 0.5 M KCl. Under acidic conditions (pH 5.8), the amount of complex degradation increases to 14% in 48 h, possibly due to a proton catalyzed hydrolysis of the macrocycle. For in vivo applications, the stability of the complex under physiological conditions (pH 7.4, 0.15 M KCl, 37 °C) is of interest. We investigated the effect of a change in each of the variables separately and found that elevation of the temperature from 25 to 37 °C produced the largest increase in complex degradation; in 48 hours 12% of the Eu^{3+} was released by the macrocycle (Figure 6). This degree of decomposition is approximately 4 times that observed by Morrow and co-workers in a recent study of the cleavage of RNA by various $Ln(HAM)^{3+}$ complexes.[3] A reduction in salt concentration from 0.5 to 0.15 M KCl produces a negligible effect (less than 1% increase), while raising the pH to 7.4 increases the degradation rate by ~1% (Table II). When all three variables are changed simultaneously to physiological conditions, almost 20% of the initial $Eu(HAM)^{3+}$ sample degrades in 48 h. From these results it is evident that the integrity of the complex is dependent on the experimental conditions employed. The pH and temperature sensitivity observed by us may account for the differences between our results

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Table II. Stability of Eu(HAM)$^{3+}$ as a Function of pH, Temperature, and KCl Concentration

pH	T (°C)	[KCl] (M)	% degradation[a]	pH	T (°C)	[KCl] (M)	% degradation[a]
7.0	25	0.5	3.7	7.4	25	0.5	4.7
5.8	25	0.5	14.0	7.4	25	0.15	5.6
7.0	25	0.15	4.3	7.4	37	0.15	19.0
7.0	37	0.5	11.8				

[a] Determined as described in the Experimental Section. All values reflect sample incubation for 48 h.

and those of other researchers.[2,3] The increased rate of Eu(HAM)$^{3+}$ decomplexation observed in our studies is likely due to the ability of the luminescence technique to detect directly and accurately small amounts of Eu^{3+}.

Gd(HAM)$^{3+}$ as a Relaxation Agent for ^{89}Y NMR. Recent work in this laboratory has focussed on developing the lanthanide-like Y^{3+} ion as a probe for the metal ion binding sites of calcium-binding proteins belonging to the calmodulin superfamily.[4] The Y^{3+} ion with its 100% natural abundance as ^{89}Y and nuclear spin of $^1/_2$, displays a calcium-like electrostatic binding, thus making it a potential probe ion for calcium-binding proteins. ^{89}Y NMR experiments are complicated by the low sensitivity of Y^{3+} (0.67 relative to ^{13}C) and long spin–lattice relaxation times (T_1 on the order of minutes). However, with the use of pulsed Fourier techniques and paramagnetic relaxation agents these disadvantages can be overcome. Holz and Horrocks[4] have recorded the ^{89}Y NMR spectrum of Y^{3+}-bound pike parvalbumin by using the relaxation agent Gd(dtpa)$^{2-}$. For extension of ^{89}Y NMR experiments to calmodulin, we sought a relaxation agent which is more exchange-inert and possesses a greater relaxivity than Gd(dtpa)$^{2-}$.

The favorable results of the Eu(HAM)$^{3+}$ stability studies and the efficient ^{1}H relaxivity of the Gd^{3+} analog reported by Smith[2] encouraged us to test the ^{89}Y relaxivity properties of Gd(HAM)$^{3+}$. Relaxivity, a measure of a paramagnetic agent's ability to catalyze the relaxation of water protons, increases with the electronic spin of the paramagnetic species and the number of water molecules coordinated to it.[25] The spin of the unpaired electrons, when coupled with molecular tumbling in solution, induces fluctuations in local magnetic fields which provide a pathway by which the water protons can relax. In a comparison of relaxivity induced by Gd^{3+} complexes of various acyclic and macrocyclic polyamino carboxylic acids, Chang[18,26] observed a linear correlation between the number of metal ion-coordinated water molecules and the measured relaxivity. If the relaxation of the ^{89}Y nucleus by the Gd^{3+} ion involves an inner sphere process, then the larger number of water molecules coordinated to the Gd^{3+} ion in the HAM complex (q = 3) over the commonly used relaxation agent Gd(dtpa)$^{2-}$ (q = 1) suggests that the former should relax the Y^{3+} ion better than the latter.

Since ^{89}Y NMR experiments on proteins require concentrated samples (50–100 mg/mL) and lengthy NMR data acquisition times (24–48 h) to achieve good S/N, we employed small metal chelate complexes as models for the protein metal-ion binding sites. The polyamino carboxylate ligand dtpa was used as the protein model for a comparison of the ^{89}Y relaxivity of Gd(HAM)$^{3+}$ and Gd(dtpa)$^{2-}$. In separate experiments, Gd(HAM)$^{3+}$ was observed to reduce the spin–lattice relaxation time of the Y^{3+} ion bound to the polyamino carboxylic acid ligands edta, egta ((ethylene glycol bis(β-aminoethyl) ether)-*N,N,N',N'*-tetraacetic acid), ttha(triethylenetetraminehexaacetic acid), and nta (nitrilotriacetic acid). As expected, Gd(HAM)$^{3+}$ relaxes the Y^{3+} ion more than Gd(dtpa)$^{2-}$, as measured by S/N improvement (Figure 7). However, the more striking result is that the concentration of the macrocyclic complex necessary to

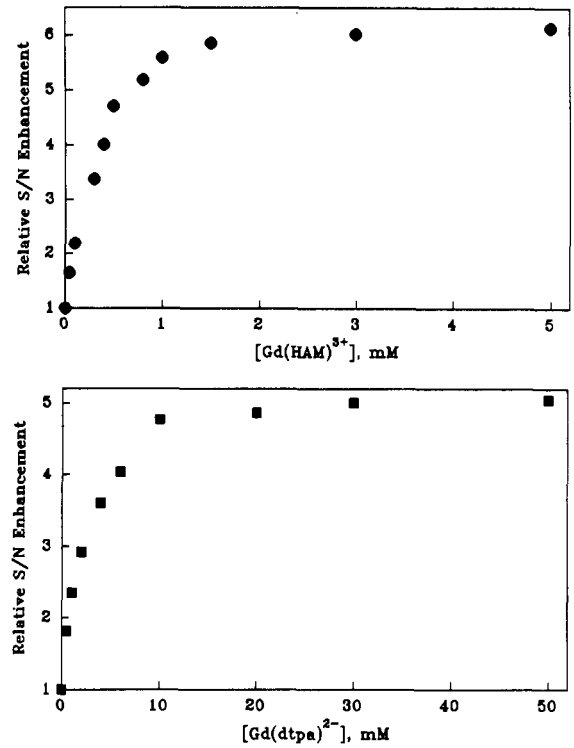

Figure 7. Enhancement of the single ^{89}Y NMR resonance of Y(dtpa)$^{2-}$ at 82.6 ppm as a function of added (●) Gd(HAM)$^{3+}$ or (■) Gd(dtpa)$^{2-}$. Values reported are relative to the S/N obtained in the absence of added relaxation agent.

reach the maximum relaxivity is one order of magnitude less for Gd(HAM)$^{3+}$ than for Gd(dtpa)$^{2-}$. Considering the fact that Gd(HAM)$^{3+}$ relaxes water protons better than the Gd^{3+}_{aq} ion[2] (q = 9), the number of water molecules bound to the Gd^{3+} ion may not be the sole factor determining the relaxivity (which exhibits field and temperature dependencies) for Gd(HAM)$^{3+}$. A more important aspect may be that Gd(HAM)$^{3+}$ and Gd(dtpa)$^{2-}$ are oppositely charged. The electrostatic attraction between the cationic Gd(HAM)$^{3+}$ and anionic Y(dtpa)$^{2-}$ complexes is expected, on average, to bring these two complexes into closer proximity. The r^{-6} dependence in dipolar interactions requires that relaxivity increase the closer the Y^{3+} ion approaches the Gd^{3+} ion, which is the center of unpaired spin density.[25] In addition, the Gd^{3+} ion in the nearly planar HAM molecule is more accessible for interaction with the Y^{3+} ion than the Gd^{3+} ion in the dtpa complex in which the ligand "wraps" around the metal ion.[27] With regard to our intended ^{89}Y NMR protein studies, the effect on the observed relaxivity due to either of these factors will be in the same direction. The metal-ion binding sites of calcium-binding proteins are typically rich in negatively charged residues (aspartic and glutamic acid), while the accessibility of the metal ion is a consideration for a bulky protein molecule.

Concluding Remarks. The thorough characterization of Eu(HAM)$^{3+}$ using laser-induced luminescence provides insight into the spectroscopic behavior of the Eu^{3+} ion ligated primarily by nitrogen atoms. At a first glance, the luminescent properties of the molecule appear typical; however, further investigation reveals pH sensitive changes in the excitation spectrum and the excited state lifetime of the Eu^{3+} ion in the complex. The observation of two species in solution in intermediate exchange is a seldom observed event in Eu^{3+} luminescence studies.

The sensitivity of the luminescence technique allows for the direct detection of uncomplexed Eu^{3+} and evaluation of complex stability. While the amount of Eu(HAM)$^{3+}$ degradation determined by the luminescence technique is greater than those previously reported,[2,3] the molecule still possesses a remarkable

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stability and inertness in aqueous solution, remaining intact even in the presence of a strong chelating agent or a competing metal ion. The utility of $Ln(HAM)^{3+}$ complexes as in vivo probe molecules ultimately depends on the necessary experimental conditions and the toxicity of the free metal ion and/or the free ligand relative to the initial amount administered. Results of our stability studies indicate strong temperature and pH dependencies which should be considered when identifying uses for the macrocyclic complex.

The stepwise deprotonation of the water molecules bound to the Eu^{3+} ion suggests that the relaxivity of the Gd^{3+} analog may also be pH dependent. In fact, a study of the pH-induced dimerization of Gd(hedta)[28] indicates that dimerization of the complex under alkaline conditions does not compromise the relaxation efficiency of the Gd^{3+} ion, but increases it approximately by a factor of 2. Experiments designed to evaluate the relaxivity of $Gd(HAM)^{3+}$ as a function of pH may provide further insight into the factors responsible for the relaxation properties of the molecule.

The ^1H NMR[2] and preliminary ^{89}Y NMR results indicate that $Gd(HAM)^{3+}$ possesses the ability to relax these nuclei effectively. The solubility of HAM in both aqueous and organic solvents renders it a potential relaxation agent for other nuclei (e.g. ^{13}C) as well. Experiments which examine the relaxation of protein-bound Y^{3+} ions by $Gd(HAM)^{3+}$ are currently in progress.

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