Displacement of Inner-Sphere Water Molecules from Eu^{3+} Analogues of Gd^{3+} MRI Contrast Agents by Carbonate and Phosphate Anions: Dissociation Constants from Luminescence Data in the Rapid-Exchange Limit

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Europium(III) $^7F_0 \rightarrow {}^5D_0$ excitation spectroscopy is used to determine if the anions carbonate and phosphate present in physiological fluids are able to displace water molecules from the first coordination sphere of Eu^{3+} analogues of Gd^{3+} MRI contrast agents. A lengthening of the Eu^{3+} excited state lifetime in the presence of millimolar concentrations of carbonate or phosphate indicates that water molecules are displaced by an anion. Only those metal complexes that contain negatively charged ligands and more than one water molecule in the first coordination sphere of Eu^{3+} have their water molecules displaced by saturating concentrations of carbonate or phosphate. Conditional dissociation constants, K_d's, for Eu^{3+}−ligand complexes with phosphate or carbonate are determined from titrations wherein the Eu^{3+} excited state lifetimes are monitored. For phosphate, K_d's lie in the range 1.2−90 mM, whereas for carbonate, the range is 35−200 mM. The titrations also indicate that only a single anion binds to a metal chelate complex and that the single anion may, under saturating anion concentrations, displace on average more than one, but not all, first coordination sphere water molecules. Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ excitation spectra indicate that, in some cases, many different Eu^{3+}-containing species are in fast exchange in the presence of added anion, presumably involving different numbers of first coordination sphere water molecules. Our results show that, under physiological conditions, phosphate and carbonate will, on average, displace less than half of a water molecule from the first coordination sphere of a typical contrast agent and suggest that the effect on proton spin relaxation is likely to be minimal.

Introduction

Contrast agents in magnetic resonance imaging (MRI) consisting of a paramagnetic metal ion (usually Gd^{3+}) encapsulated by a ligand provide contrast by shortening the proton relaxation times of water molecules in the vicinity of the agent compared to water molecules in other areas of the body.[1−3] The reduction of the proton relaxation time of bulk water per unit millimolar concentration of contrast agent is defined as the relaxivity of an MRI contrast agent. The relaxivity of a contrast agent scales linearly with the number of water molecules, q, that are in the first coordination sphere of the agent's metal ion.

One of many approaches to develop contrast agents with higher relaxivities has been to create complexes with larger q-values, producing more paramagnetically affected water molecules to exchange with the bulk solvent. An important practical consideration of this design approach is the possible displacement of ligated water molecules from the first coordination sphere of the metal ion by exogenous molecules or ions, potentially lowering the relaxivity of the contrast agent. Ternary complexes have been shown to exist between anions[4−11] such as fluoride, phosphate, and carbonate and lanthanide metal complexes not used as MRI contrast agents. Burai et al.[12] reported ternary complex formation between Gd^{3+} contrast agents and the physiologically-relevant anions carbonate, phosphate, and citrate on the basis of differences between the potentiometric titration curves of an anion obtained with and without a Gd^{3+}−ligand complex present. Our goal in the present research is to ascertain to what extent inner-sphere water molecules are displaced from Eu^{3+} analogues of Gd^{3+} contrast agents by the physiologically present anions phosphate and carbonate and determine what properties of the complexes are likely to inhibit the formation of these ternary complexes. Further, we introduce a method for determining dissociation constants from the measurement of luminescent decay lifetimes for Eu^{3+} complexes which are in rapid exchange.[13] We use the proven method of Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ luminescence spectroscopy[3,14−17] to probe directly the first coordination sphere of the

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metal ion in Eu^{3+}−ligand complexes of various *q*-values and overall charges in the presence of carbonate and phosphate. Since Gd^{3+} and Eu^{3+} are similar in size and coordination chemistry,[18−20] the results obtained on the Eu^{3+} complexes provide significant insight into ternary complex formation by analogous Gd^{3+} complexes which are used as MRI contrast agents.

It is well-known that the difference between the excited state Eu^{3+} luminescence decay rates in H$_2$O ($\tau^{-1}_{H_2O}$) and D$_2$O ($\tau^{-1}_{D_2O}$) solution is directly proportional to the number of water molecules, *q*, in the first coordination sphere of the europium ion.[21−24] This difference in rates can therefore be used to determine *q*-values according to eq 1, where n_{OH} is the number

$$q = A(\tau^{-1}{}_{H_2O} - \tau^{-1}{}_{D_2O} - k_{XH}) \tag{1a}$$

$$k_{XH} = \alpha + \beta n_{OH} + \gamma n_{NH} + \delta n_{O=CNH} \tag{1b}$$

of alcoholic O−H oscillators in the first coordination sphere of Eu^{3+}, n_{NH} is the number of secondary amine N−H oscillators in the first coordination sphere of Eu^{3+}, and $n_{O=CNH}$ is the number of amide N−H oscillators in which the amide carbonyl oxygen is in the first coordination sphere of Eu^{3+}. The respective contributions of these X−H oscillators to the quenching of the $^5D_0$ state of Eu^{3+} are $\beta^{25} = 0.45$ ms^{-1}, $\gamma = 0.99$ ms^{-1} (calculated as the average of 0.759,[26] 1.0,[27] and 1.2[22]), and $\delta^{22,28} = 0.075$ ms^{-1}. The quantity α is the Eu^{3+} $^5D_0$ deexcitation rate constant caused by solvent water molecules in the second coordination sphere and beyond, sometimes called “closely diffusing” water molecules.[22,28] Work in this laboratory[21] on crystalline solids containing known numbers of coordinated water molecules led to the original value of the proportionality constant *A* of 1.05 waters·ms with $\alpha = 0$. Recently, Beeby et al.[22] suggested a value of $\alpha = 0.25$ ms^{-1} and proposed a new value for *A* of 1.2 waters·ms. Unfortunately they used only complexes of $q \leq 1$ to find these values which we find overestimate *q*-values in complexes of larger *q*-values (for example, the Eu(dipicolinate)$^+$ complex,[14] which has a *q*-value of 6, is predicted to have $q = 6.4$ using the Beeby et al.[22] formula). We find[29] that the values $A = 1.11$ waters·ms and $\alpha = 0.30$ ms^{-1} give a standard error in *q* of only ±0.1 and produce more reasonable predictions of *q* than do the parameters of Beeby et al.[22] over the entire range of possible *q*-values.

Experimental Section

Materials. EuCl$_3$·6H$_2$O, edta [ethylenediaminetetraacetic acid], dtpa [diethylenetriaminepentaacetic acid], hedta [*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid] (Aldrich Chemical Co.), Eu(abhc)$^{3+}$ [(1,7-bis(carbamoylmethyl)-4,10-bis(hydroxyethyl)-1,4,7,10-tetraazacyclododecane)europium(III)][30] (a gift from Janet Morrow of SUNY at Buffalo), do3a [1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid], dota [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid], dtpa-bma [*N1*,*N3*-bis(methylamine)diethylenetriaminetriacetic acid] (gifts from the now defunct Nycomed Salutar, Inc.), anhydrous sodium hydrogen carbonate [NaHCO$_3$], and anhydrous sodium monohydrogen phosphate [Na$_2$HPO$_4$] (J. T. Baker) were from the sources noted. All other chemicals were obtained from standard commercial sources and were used as received. The ligand nota [1,4,7-triazacyclononane-1,4,7-triacetic acid] was synthesized by literature methods.[31] All water used was singly distilled and passed through a NANOpure deionization system.

Methods. The concentrations of the Eu^{3+} stock solutions (ca. 20 mM) were accurately determined by edta titrations using arsenazo I as the indicator.[32] The concentrations of ligand stock solutions were determined by titrating diluted aliquots with a standardized Eu^{3+} solution and monitoring the intensity of the Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ excitation peak corresponding to the metal complex (stoichiometric 1:1 Eu^{3+}:ligand binding is observed in all cases). Five hundred micromolar Eu^{3+}−ligand complex stock solutions with all studied ligands were prepared by adding a 10% excess of ligand to Eu^{3+} and diluting to volume with H$_2$O. Care was taken that the Eu^{3+}−ligand complex stock solutions contained no free Eu^{3+}(aq) as judged by a Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ excitation scan. Owing to the slow kinetics of the formation of the Eu(dota)$^-$ complex,[33] the mixture of Eu^{3+} with dota was allowed to reach equilibrium for at least 24 h before the addition of phosphate or carbonate. HEPES and phosphate buffer stock solutions were prepared by adding weighed amounts of solid to water and adjusting the pH to 7.4 with HCl or NaOH solutions (DCl or NaOD was used with D$_2$O solutions) and diluting to volume. Carbonate solutions were prepared similarly with NaHCO$_3$, except that solutions at pH 7.4 also contained 50 mM HEPES. Because of the slow loss of aqueous carbonate due to the evolution of CO$_2$(g) at pH 7.4, the concentration of carbonate was periodically checked gravimetrically by precipitating the carbonate as its barium salt from an aliquot of the stock solution. All carbonate concentrations are believed to be accurate to within ±10%.

Eu^{3+} excitation spectra of the $^7F_0 \rightarrow {}^5D_0$ transition (578−581 nm) and excited $^5D_0$ state lifetimes were obtained using a tunable Continuum TDL-50 dye laser pumped by a YG-581C Q-switched Nd:YAG laser (10 Hz, 40−70 mJ/pulse) described elsewhere.[34,35] The $^5D_0 \rightarrow {}^7F_2$ emissive transition at 614 nm was monitored for both Eu^{3+} excitation spectra and lifetime determinations.

Titration of phosphate or carbonate into a Eu^{3+} chelate complex was accomplished by preparing a series of 1 mL solutions, usually 20 μM Eu^{3+} chelate and 0−400 mM anion, and allowing them to sit at least 5−10 min before measurements were performed at a thermostated 25 °C.

The determination of Eu^{3+} excited state lifetimes was achieved by fitting Eu^{3+} luminescence decay data to mono- or multiexponential decay functions. Fitting of titration data to the appropriate equation (*vide infra*) permits the determination of the anion dissociation

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Table 1. Eu^{3+} Excited State Lifetimes of Eu^{3+}−ligand Complexes[a]

complex	τ_{H_2O} (μs)	τ_{D_2O} (ms)	q_{calc}	τ (μs) 0.45 M phosphate	τ (μs) 0.45 M carbonate
$Eu(dota)^-$	640	2.4	0.94	644	666
$Eu(dtpa)^{2-}$	632	2.6	1.0	640	652
Eu(do3a)	290	2.2	1.9	409	458
$Eu(edta)^-$	320	2.6	2.7	519	550[b]
Eu(dtpa-bma)	592	2.5	0.93	602	605
Eu(hedta)	263	2.2	2.9	436	443
Eu(nota)	287	1.7	2.9	594[c]	505[b]
$Eu(abhc)^{3+}$	341	1.5[d]	0.85	416	411

[a] All samples are at pH 7.4 and 25 °C and contain 0.15 M NaCl. All lifetimes, τ's, are known to ±2%. [b] These luminescence decay fits also required a small component in the range of 250 μs, most likely due to the formation of a Eu^{3+}−carbonate complex seen at 579.0 nm. [c] Does not fit well to a single exponential. [d] From ref 30.

constants. The program Peakfit (SPSS Inc.) was used for these nonlinear regression fits.

Results and Discussion

Of the eight complexes studied, Eu(do3a), $Eu(edta)^-$, Eu(hedta), Eu(nota), and $Eu(abhc)^{3+}$ had their lifetimes significantly lengthened in the presence of 0.45 M phosphate or carbonate as shown in Table 1. The results in Table 1 are all obtained at the physiological pH of 7.4, although the same effect is seen up to pH 10 (data not shown). The lengthening of the Eu^{3+} excited state lifetime in the presence of added anions is due to the replacement of water molecules in the first coordination sphere of Eu^{3+} by carbonate or phosphate anions. Furthermore, it is only the same five complexes which have new, red-shifted bands in their Eu^{3+} excitation scans (Figure 1), which is expected if additional carbonate or phosphate oxygens coordinate to the europium ion.[36] For each system, luminescence decays obtained with excitation at these various excitation maxima all reveal identical lifetimes (Table 1), indicating that the Eu^{3+}−ligand complex and the Eu^{3+}−ligand−anion complex(es) are in rapid exchange.[13]

To determine the affinities of the anions for the various Eu^{3+} chelates at pH 7.4, each complex was titrated separately with phosphate or carbonate. The titration curves (observed luminescence decay rate constants, k_{obs}, vs anion concentration) are shown in Figure 2. The titration curves were fit to eq 2, which

$$F = [A]_{tot} + [ML]_{tot} + \boldsymbol{K_d} \tag{2a}$$

$$[MLA] = \frac{F - \sqrt{F^2 - 4[ML]_{tot}[A]_{tot}}}{2} \tag{2b}$$

$$X_{MLA} = [MLA]/[ML]_{tot} \tag{2c}$$

$$X_{ML} = 1 - X_{MLA} \tag{2d}$$

$$k_{obs} = 1/\tau \tag{2e}$$

$$k_{obs} = X_{ML}\boldsymbol{k_{ML}} + X_{MLA}\boldsymbol{k_{MLA}} \tag{2f}$$

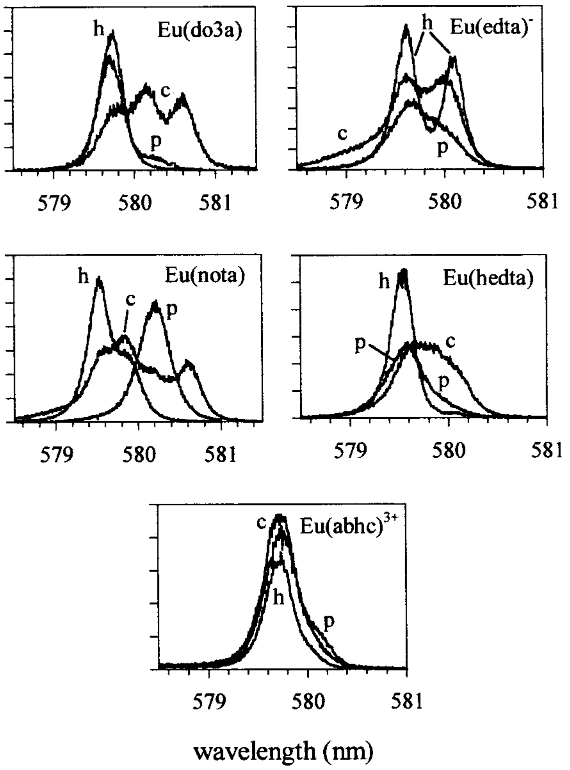

Figure 1. Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ excitation spectra of the indicated metal complexes containing either 50 mM HEPES (labeled h), 0.45 M phosphate (labeled p), or 50 mM HEPES and 0.45 M carbonate (labeled c). All samples are at pH 7.5 and 25 °C and contain 0.15 M NaCl and 10−20 μM Eu^{3+}−ligand complex.

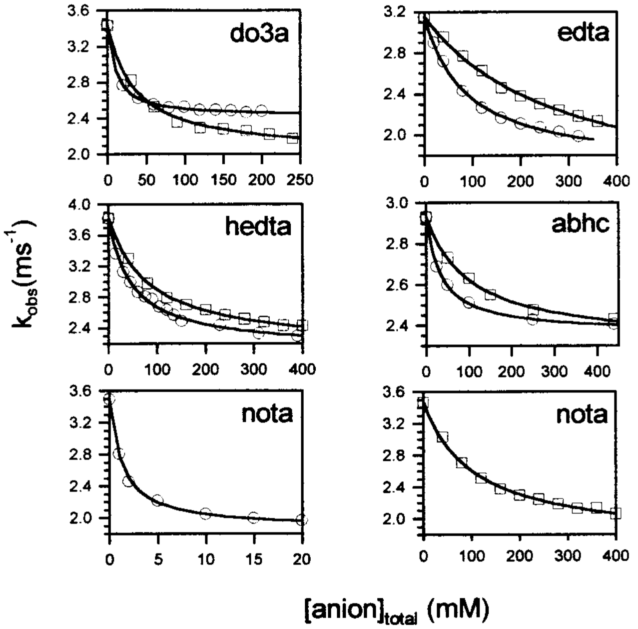

Figure 2. Titration curves (observed luminescence decay rate constants, k_{obs}, vs total added anion concentration) of the indicated Eu^{3+} chelates with phosphate (circles) and carbonate (squares). The solid curves represent the best fits to eq 2. Samples are at pH 7.5 and 25 °C and contain 0.15 M NaCl and 20 μM Eu^{3+}chelate.

models a single anion binding to a single metal complex. Here A is the studied anion, ML is the Eu^{3+}−ligand complex, MLA is the ternary Eu^{3+}−ligand−anion complex, X_Y is the mole fraction of species Y, k_Y is the excited Eu^{3+} luminescence decay rate constant of species Y, and k_{obs} is the observed luminescence decay rate constant of the excited Eu^{3+} in the system. Charges have been omitted in eq 2 for clarity. The boldface quantities are the parameters of the fit. The results of fitting the titration data to eq 2 are given in Table 2. It should be noted that the dissociation constants, K_d's, obtained from eq 2 are conditional dissociation constants for the ternary Eu^{3+}−ligand−anion

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Table 2. Results of the Fits to Eq 2 of Data for Eu^{3+}−ligand Complex Titrations with Anions[a]

complex	K_d (mM) for MLA	τ_{MLA} (μs)	q_d	q_d(phys)[d]
		Anion: Phosphate		
Eu(do3a)	10.2	414	1.1	0.3
$Eu(edta)^-$	86.9	604	1.6	0.07
Eu(hedta)	49.3	472	1.9	0.1
Eu(nota)[b]	1.25	536	1.8	1.4
$Eu(abhc)^{3+}$	34.6	423	0.6	0.07
		Anion: Carbonate		
Eu(do3a)	35.3	499	1.6	0.6
$Eu(edta)^-$ [c]	298	788	2.1	0.1
Eu(hedta)	81.8	469	1.9	0.4
Eu(nota)	106	591	2.0	0.3
$Eu(abhc)^{3+}$	102	434	0.7	0.1

[a] Parameters: τ_{MLA}, lifetime of the MLA complex at saturating anion concentration; q_d, number of waters displaced by A at saturating anion concentration; q_d(phys), waters displaced by anion at physiological anion concentration. [b] Titration curve calculated from the predominant lifetime (>90%) fit to the Eu^{3+} excited state decay. [c] The titration curve does not exhibit adequate curvature to produce a unique fit to eq 2. Higher carbonate concentrations needed to remedy this situation were prohibited due to solubility problems; therefore the parameters obtained from the fit must be viewed with caution and simply indicate weak binding of carbonate to the complex. [d] Calculated using [phosphate] = 4 mM, [carbonate] = 20 mM, and $[ML]_{tot} = 20\ \mu M$.

complex interactions measured at pH 7.4. The excellent fit of the data to eq 2 (coefficients of determination, r^2, ≥ 0.993) is good evidence that only one phosphate or carbonate anion binds to each Eu^{3+}−ligand complex.

A common feature of four of the five Eu^{3+}−ligand complexes which have inner-sphere water molecules displaced by carbonate and phosphate is that they possess more than one coordinated water molecule ($q > 1$), suggesting that the anions, which are larger than water molecules, are kept from displacing the single inner-sphere water molecule in complexes of $q = 1$ owing to steric effects. Indeed Burai et al.[12] conclude that any Gd^{3+} complex with water molecules bound to solvent-exposed metal sites which are cis to one another will bind phosphate or carbonate at pH 7.4. However, these anions form stable ternary complexes with $Eu(abhc)^{3+}$ which has a q-value of 1. Thus, in addition to steric repulsion, electrostatic repulsion of the anions from the solvent-exposed metal sites by negative metal-binding moieties of the ligand must be considered as a factor in the displacement of water molecules by anions. Furthermore, the lack of formation of complexes of higher order than ternary suggests that once a single anion binds to a Eu^{3+}−ligand complex, the larger negative charge around the remaining solvent-exposed site prevents the binding of a second anion to the metal center.

Although only a single anion binds to a Eu^{3+} complex, that anion may displace more than one water molecule. The number of water molecules displaced by saturating concentrations of anion, q_d, can be found by substituting the lifetime of the Eu^{3+} luminescence at saturating anion concentrations, τ_{MLA}, for τ_{D_2O} in eq 1 and setting $k_{XH} = 0$. Indeed, Dickins et al.[4] have suggested the bidentate binding of carbonate to a Eu^{3+}−ligand complex of $q = 2$ with the displacement of two water molecules, and many of the q_d values in Table 2 are close to 2. However, the facts that most of the q_d values are less than 2 and that multiple red-shifted peaks are seen in the Eu^{3+} excitation scans of certain ternary systems, especially the carbonate complexes of Eu(do3a) and Eu(nota), indicate that bidentate carbonate binding may be only one of several interconverting binding motifs for that ligand.

Unlike the ability to form a ternary complex, the actual affinity (K_d values) of an anion for a particular chelate complex is not well predicted by the q-value and the negative charge around the solvent-exposed metal ion. A case in point is Eu(nota), which has many properties similar to those of Eu(hedta) yet forms an anomalously stable ternary complex with phosphate. The luminescence decay of Eu(nota) in the presence of phosphate does not well conform to a single-exponential decay because of the slow exchange between Eu(nota) and Eu(nota)−phosphate complexes.[13] The only trends that can be seen in the measured K_d's are that the overall negative $Eu(edta)^-$ complex has the largest of the measured K_d values (weakest association) for either anion and that carbonate in all cases interacts more weakly with the Eu^{3+}−ligand complexes than does phosphate. The K_d values may be dependent on the lability of the anion−metal bond and be sensitive to any particularly strong H-bonds formed between the ligand and anion.

Finally, and quite importantly, even though high millimolar concentrations of phosphate and carbonate displace water in Eu^{3+} complexes with q values >1, the effect is not dramatic under physiological conditions. As seen in Table 2, at physiological carbonate and phosphate concentrations in blood plasma (20 and 4 mM respectively[37]), these anions displace, on average, at most half of a water molecule from the complexes (excluding the Eu(nota) complex). Nonetheless, *in vitro* relaxivity studies should be performed in the presence of physiological concentrations of phosphate and carbonate to provide an accurate prediction of their *in vivo* relaxivities.

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