Eu(III) Macrocyclic Complexes Promote Cleavage of and Bind to Models for the 5′-Cap of mRNA. Effect of Pendent Group and a Second Metal Ion

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Received October 12, 1999

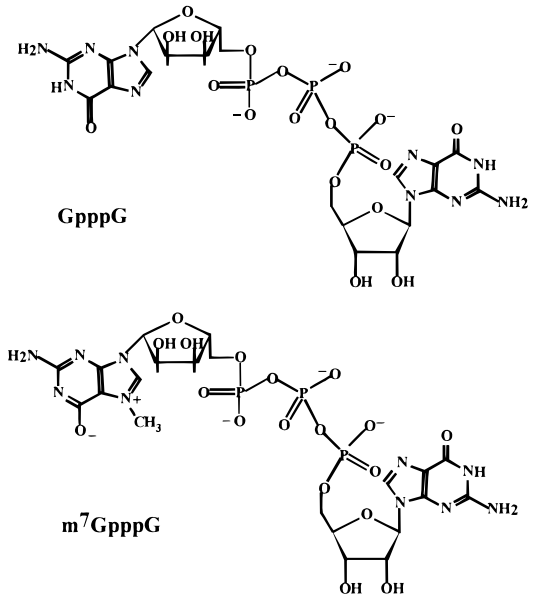
The interaction of three Eu(III) macrocyclic complexes Eu(THED)$^{3+}$, Eu(ATHC)$^{3+}$, and Eu(S-THP)$^{3+}$ with two 5′-cap model compounds, GpppG and m^{7}GpppG is studied (THED = 1,4,7,10-tetrakis(2-hydroxyethyl)-1,4,7,-10-tetraazacyclododecane, ATHC = 1-(carbamoylmethyl)-4,7,10-tris(2-hydroxyethyl)-1,4,7,10-tetraazacyclododecane, S-THP = 1S,4S,7S,10S-tetrakis(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane). Laser-induced excitation luminescence spectroscopy is used to study the binding of Eu(S-THP)$^{3+}$ to GpppG ($K = 5.9 \times 10^4$ M^{-1}) and to characterize the Eu(S-THP)−GpppG complex. Both Eu(THED)$^{3+}$ and Eu(S-THP)$^{3+}$ bind to m^{7}GpppG as monitored by use of fluorescence spectroscopy with binding constants of 5.9×10^3 and 4.4×10^4 M^{-1}, respectively. The kinetics of cleavage of GpppG by two macrocyclic complexes is studied. Cleavage of GpppG by Eu(THED)$^{3+}$ is accelerated by 15-fold in the presence of an equivalent of $Zn(NO_3)_2$ at pH 7.3, 37 °C, suggesting that two metal ions accelerate the cap cleavage reaction. Eu(ATHC)$^{3+}$ promotes cleavage of GpppG with a pseudo-first-order rate constant of 2.6×10^{-5} s^{-1} at pH 7.3, 37 °C, and 0.30 mM complex.

Introduction

The 5′-ends of RNAs that are synthesized by RNA polymerase II are modified with a structure called the 5′-cap. The 5′-cap structure contains a N7-methylated guanosine linked through triphosphate to the 5′-terminius of the RNA. The 5′-cap is important in several processes in mRNA metabolism including splicing, translation, and stabilization of the mRNA.[1] Because of the importance of the 5′-cap structure in RNA metabolism, inactivation of the mRNA transcript through destruction of the 5′-cap has been investigated as a strategy to selectively inhibit gene expression at the mRNA level.[2]

Previous research demonstrated that Cu(II) complexes hydrolyze the triphosphate linkage of the 5′-cap structure at physiological temperature and pH.[3,4] Eu(III) macrocyclic complexes are even more effective at promoting cleavage of the 5′-cap both as free complexes and as complexes attached to antisense oligonucleotides.[2,5] However, cleavage of the 5′-cap by both Cu(II) and Eu(III) complex−oligonucleotide conjugates is slow relative to mRNA turnover in cells. Our interest in the development of more potent catalysts[2,5−7] has led us to study the interaction of metal ion complexes with the 5′-cap structure and to extend our studies of the kinetics of cleavage by Eu(III) complexes.

Chart 1

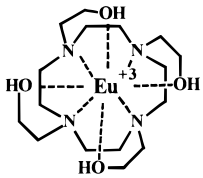


Here, we describe the interaction of three Eu(III) macrocyclic complexes Eu(THED)$^{3+}$, Eu(ATHC)$^{3+}$, and Eu(S-THP)$^{3+}$ with two 5′-cap model compounds GpppG and m^{7}GpppG (Charts 1 and 2). Our goal was to study the binding of Eu(III) macrocyclic complexes to the 5′-cap analogues in order to rationalize results from kinetics studies[5] and to determine some of the first binding constants for these substrates to mononuclear metal ion complexes. Toward the development of more active catalysts, cleavage of GpppG by Eu(THED)$^{3+}$ is studied in the presence of a second metal to lay the groundwork for the development of dinuclear catalysts.[6,7] Finally, a Eu(III) macrocyclic complex containing mixed amide and alcohol pendent groups is studied

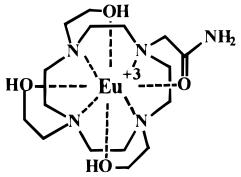
(1) Baker, B. F. *Antisense Research and Applications*; Crooke, S. T., LeBlue, B., Eds.; CRC Press: Boca Raton, FL, 1993; pp 37−54.
(2) Baker, B. F.; Lot, S. S.; Kringel, J.; Cheng-Flournoy, S.; Villiet, P.; Sasmor, H. M.; Siwkowski, A. M.; Chappell, L. L.; Morrow, J. R. *Nucleic Acids Res.* **1999**, *27*, 1547−1551.
(3) Baker, B. F. *J. Am. Chem. Soc.* **1993**, *115*, 3378−3379.
(4) Baker, B. F.; Ramasamy, K.; Kiely, *J. Bioorg. Med. Chem. Lett.* **1996**, *6*, 1647−1652.
(5) Baker, B. F.; Khalili, H.; Wei, N.; Morrow, J. R. *J. Am. Chem. Soc.* **1997**, *119*, 8749−8755.
(6) McCue, K. P.; Voss, D. A., Jr.; Marks, C.; Morrow, J. R. *J. Chem. Soc., Dalton Trans.* **1998**, 2961−2964.
(7) McCue, K. P.; Morrow, J. R. *Inorg. Chem.* **1999**, *38*, 6136−6142.

10.1021/ic9912068 CCC: $19.00
Published on Web 04/25/2000

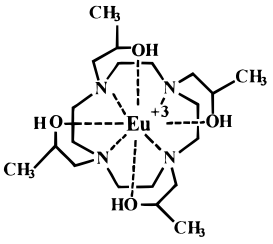
Chart 2



Eu(THED)$^{3+}$



Eu(ATHC)$^{3+}$



Eu(S-THP)$^{3+}$

as a promoter for cleavage of GpppG. These complexes are of interest[8] because they have been attached to oligonucleotides to promote sequence-selective "decapitation" of an mRNA.[2]

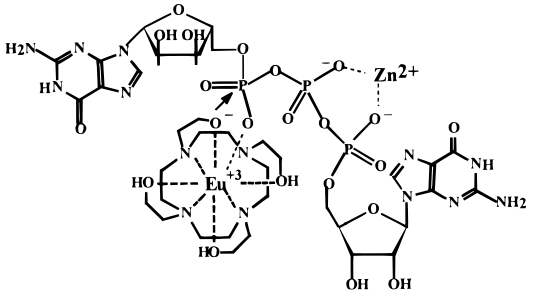
Experimental Section

The capped monoribonucleotides m^{7}GpppG and GpppG were purchased as sodium salts from Amersham Pharmacia Biotech and concentrations were determined by UV−vis absorbance using ϵ = 18 800 M^{-1} cm^{-1} at 250 nm and ϵ = 19 100 M^{-1} cm^{-1} at 252 nm for m^{7}GpppG and GpppG, respectively. Eu(ATHC)$^{3+}$, Eu(THED)$^{3+}$, and Eu(S-THP)$^{3+}$ were prepared as reported.[8−10] Buffers and nucleotide standards were purchased from Sigma and used as received.

m^{7}GpppG Luminescence. m^{7}GpppG fluorescence was measured on an SLM-Aminco 8100 spectrofluorometer with a MC200 monochromator for emission and MC400 monochromator for excitation. A 5 mm × 5 mm fluorescence cell was used. The samples were kept in a constant temperature cell holder, and a Brinkman RMG Lauda water bath controlled the temperature. The wavelength for excitation was 280 nm, and fluorescence emission intensity was measured at 360 and 384 nm. Titrations were carried out with 10 μM m^{7}GpppG and 40 mM MES at pH 6.5 or with 40 mM HEPES at pH 7.5. Solutions of Eu(THED)$^{3+}$ or Eu(S-THP)$^{3+}$ containing 10% EDTA were added via pipet. For a typical titration, concentrations ranged from 1.0 × 10^{-5} to 1.8 × 10^{-3} M for Eu(THED)$^{3+}$ or up to 5.0 × 10^{-4} M for Eu(S-THP)$^{3+}$. Emission intensities were corrected for dilution because of added titrant. Because of the strong binding of Eu(S-THP)$^{3+}$ and the low ratios of complex to m^{7}GpppG used initially, it was necessary to take into account complex formation when determining the concentration of free Eu(S-THP)$^{3+}$ (see Results for details).[11]

Eu(III) Luminescence Spectroscopy. 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.[12,13] The $^7D_0 \rightarrow {}^7F_2$ emissive transition at 614 nm was monitored for both Eu(III) excitation spectra and lifetime determinations.

Scheme 1



Binding of GpppG to Eu(S-THP)$^{3+}$ was measured by adding GpppG (final concentration of 10 to 134 μM) to a solution containing either 20 μM Eu(S-THP)$^{3+}$ and 50 mM MES at pH 6.5 or 50 mM HEPES at pH 8.0. Excitation spectra were acquired from 578 to 580.5 nm, and emission at 614 nm was recorded. The data were fit using Peakfit, and the area of the peak attributed to Eu(S-THP)-GpppG was determined.

The number of water molecules bound to Eu(III) in the Eu(S-THP)$^{3+}$ and Eu(S-THP)−GpppG complexes was determined by luminescence lifetime measurements in H_2O and D_2O at pH and pD 6.5.[14,15] Values for the lifetime of Eu(S-THP)−GpppG were measured under conditions in which the bound form was present in excess and excitation was carried out at a wavelength (579.65 nm) where only the Eu(S-THP)−GpppG complex absorbs. Solutions contained 50 mM MES, 20 μM Eu(S-THP)$^{3+}$, and 20 μM GpppG at 10 °C.

Kinetics of Cleavage of GpppG. The rate of GpppG cleavage was determined by monitoring the disappearance of GpppG by use of a Waters 600E HPLC equipped with a 490E UV−vis detector. The dinucleotide ApA was used as an internal standard. Reaction products were identified by comparison to authentic samples of nucleotides. Reactions were analyzed on a C18 column (250 mm × 4.6 mm). The reaction mixture was eluted with an isocratic flow (1.5 mL/min) of a 1:1 mixture of solvent A (50 mM KH_2PO_4, 5 mM tetrabutylammonium phosphate at pH = 5.0) and solvent B (1:1 methanol/100 mM KH_2PO_4, 10 mM tetrabutylammonium dihydrogen phosphate) for 30 min. Reaction mixtures contained 0.030 mM GpppG, 0.30 mM Eu(ATHC)$^{3+}$ or Eu(THED)$^{3+}$, 50 mM HEPES (pH = 7.3, 37 °C), and ApA (0.1 mg/mL). Aliquots of the reaction mixture were drawn at time intervals, solvent A was added, and the mixture was injected onto the column. In the absence of a metal complex, 3% of the GpppG was hydrolyzed over a period of 5 days at pH 7.3, 37 °C.

Results

Cleavage of GpppG. Cleavage of GpppG by Eu(THED)$^{3+}$ produces GMP, GDP, and a phosphate diester or a new phosphoric anhydride resulting from attack of an alcohol pendent group at either the α or β phosphate group of GpppG.[5] The primary pathway is attack at an α-phosphate to give GDP and Eu(THED)−GMP (Scheme 1). Cleavage is first order in GpppG and Eu(THED)$^{3+}$; a pseudo-first-order rate constant of (5.3 ± 1.0) × 10^{-5} s^{-1} for the 0.31 mM complex and 0.030 mM GpppG at pH 7.4, 37 °C has been reported.[5] Eu(THED)$^{3+}$ dissociates slowly in the presence of divalent metal ions with a half-life of several days at 37 °C.[10] The kinetic inertness of Eu(THED)$^{3+}$ to dissociation makes it possible to carry out experiments with Eu(THED)$^{3+}$ and other metal ions without exchange of the THED macrocycle between metal ions.

(8) Morrow, J. R.; Chin, K. A. *Inorg. Chem.* **1993**, *32*, 3357−3361.
(9) Chin, K. O. A.; Morrow, J. R.; Lake, C. H.; Churchill, M. R. *Inorg. Chem.* **1994**, *33*, 656−664.
(10) Chappell, L. L.; Voss, D. A., Jr.; Horrocks, W. D., Jr.; Morrow, J. R. *Inorg. Chem.* **1998**, *37*, 3989−3998.
(11) Conners, K, *Binding Constants*; John Wiley and Sons: New York, 1987.
(12) Frey, S. T.; Chang, C. A.; Carvalho, J. F.; Varadarajan, A.; Schultze, L. M.; Pounds, K. L.; Horrocks, W. D., Jr. *Inorg. Chem.* **1994**, *33*, 2882−2889.
(13) Frey, S. T. Ph.D. Thesis, The Pennsylvania State University, University Park, PA, 1994.
(14) Horrocks, W. D., Jr; Sudnick, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 334−340.
(15) Supkowski, R. M.; Horrocks, W. D., Jr. *Inorg. Chem.* **1999**, *38*, 5616−5619.

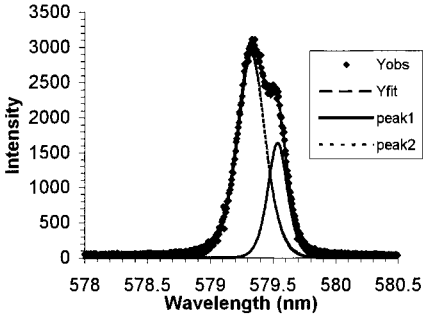


Figure 1. $^7F_0 \rightarrow {}^5D_0$ excitation spectra ($\lambda_{em} = 614$ nm) for 20 μM Eu(S-THP)$^{3+}$, with 20 μM GpppG at pH 6.5, 50 mM buffer at 10 °C.

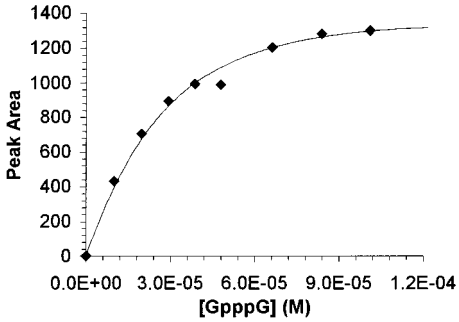


Figure 2. Peak area of the excitation peak of the Eu(S-THP)$^{3+}$−GpppG complex ($\lambda = 579.54$ nm) as a function of GpppG total concentration at pH 6.5, 10 °C, 50 mM buffer with an initial Eu(S-THP)$^{3+}$ concentration of 20 μM. Data were fit to eq 1 to give K_d of 1.7×10^{-5} M (COD = 0.991).

Zn(II) and Mg(II) were studied as catalysts for cleavage of GpppG. Neither $Zn(NO_3)_2$ (0.31 mM) nor $Mg(NO_3)_2$ (1 mM) promotes detectable cleavage of GpppG at 37 °C at pH 7.4 over a period of 1 day. However, the pseudo-first-order rate constant for cleavage of GpppG by Eu(THED)$^{3+}$ (0.31 mM) in the presence of 0.31 mM $Zn(NO_3)_2$ is $(7.8 \pm 1.2) \times 10^{-4}$ s^{-1}, which is 15-fold higher than in the absence of $Zn(NO_3)_2$. Halving the concentration of Zn(II) (0.15 mM) while maintaining the same concentrations of all other reactants halves the rate constant ($3.7 \pm 0.5) \times 10^{-4}$ s^{-1}), consistent with a reaction that is first-order in Zn(II). In contrast, 1.00 mM Mg(II) does not change the pseudo-first-order rate constant for cleavage of GpppG by Eu(THED)$^{3+}$.

A Eu(III) macrocyclic complex with one amide pendent group and three hydroxyethyl groups tethered to an oligonucleotide cleaves the 5′-cap of an mRNA by attack of an hydroxyethyl pendent group on the phosphoric anhydride linkage.[2] To ascertain the effect of an amide pendent group on cleavage rates, the kinetics of cleavage of GpppG by Eu(ATHC)$^{3+}$ was studied. Previous work showed that Eu(ATHC)$^{3+}$ is resistant to dissociation of Eu(III) both in the presence and in the absence of EDTA.[8] Eu(ATHC)$^{3+}$ promotes cleavage of GpppG at 37 °C, pH 7.3 to give GDP and GMP in a 5:1 ratio. Product ratios are similar to those observed for Eu(THED)$^{3+}$-promoted cleavage of 5′-cap analogues.[5] This suggests that the primary cleavage reaction proceeds through attack of the hydroxyethyl group of Eu(ATHC)$^{3+}$ on an α-phosphate of GpppG as observed previously[5] for Eu(THED)$^{3+}$. The pseudo-first-order rate constant for cleavage as measured by the method of initial rates for solutions containing 0.30 mM Eu(ABHC)$^{3+}$, 0.030 mM GpppG, and 0.03 mM EDTA is $(2.6 \pm 0.42) \times 10^{-5}$s^{-1}.

Binding of GpppG and m^{7}GpppG to Eu(III) Macrocyclic Complexes. Laser-induced excitation luminescence spectroscopy was used to study GpppG binding to Eu(III) macrocyclic complexes. In the absence of GpppG, the excitation spectrum of Eu(S-THP)$^{3+}$ exhibits a single peak (579.32 nm) at acidic pH values. An additional peak (577.51 nm) is present at basic pH values, which is due to either the hydroxide or alkoxide complex resulting from deprotonation of an Eu(III) water or alcohol ligand.[8] A similar spectrum is observed for Eu(THED)$^{3+}$.[8] A new excitation peak appears (579.54 nm) upon addition of GpppG to a solution containing 20 μM Eu(S-THP)$^{3+}$, 50 mM MES, pH 6.5 at 10 °C with a concomitant decrease in the intensity of the peak for Eu(S-THP)$^{3+}$ (Figure 1). The red-shifted peak is consistent with phosphate group coordination to the Eu(III) complex.[16] That the excitation spectrum of the Eu(S-THP)$^{3+}$−GpppG complex is identical at pH 8.0 and pH 6.5 suggests that a single Eu(III) species is present in this pH range.

The area under the peak at 597.54 nm that corresponds to the Eu(S-THP)$^{3+}$−GpppG adduct was plotted versus GpppG total concentration (Figure 2). The data were fit to

$$\text{peak area} = B\left[\frac{F - \sqrt{F^2 - 4[\text{Eu(S-THP)}]_{\text{total}}[\text{GpppG}]_{\text{total}}}}{2}\right] \quad (1)$$

to give a K_d of 1.7×10^{-5}. Here, K_d is a conditional dissociation constant for Eu(S-THP)−GpppG dissociating into Eu(S-THP)$^{3+}$ and GpppG. This corresponds to an equilibrium constant ($K = 1/K_d$) of 5.9×10^4 M^{-1} for the binding of GpppG to Eu(S-THP)$^{3+}$. In eq 1, B is a fluorescence proportionality constant for Eu(S-THP)−GpppG and F is defined below:

$$F = [\text{Eu(S-THP)}]_{\text{total}} + [\text{GpppG}]_{\text{total}} + K_d \quad (2)$$

The excellent fit of the data (COD = 0.991) is evidence that only the 1:1 Eu(S-THP)−GpppG complex exists.

The excitation spectrum of 20 μM Eu(THED)$^{3+}$ does not change upon addition of excess GpppG at pH 6.5, 50 mM Mes buffer at 10 °C. This is surprising because binding of Eu(THED)$^{3+}$ to m^{7}GpppG is observed under similar conditions, as described below. If we assume that the binding constants for Eu(THED)$^{3+}$ binding with GpppG and m^{7}GpppG are similar, a detectable amount of Eu(THED)−GpppG (33%) should be formed under conditions of an experiment with 20 μM Eu(THED)$^{3+}$ and 41 μM GpppG. The luminescence lifetime of the Eu(III) complex was also examined because a change in lifetime may provide evidence for interaction with GpppG.[15] The luminescence lifetime of Eu(THED)$^{3+}$ is predicted to increase upon binding of GpppG because binding of GpppG in the first coordination sphere of Eu(III) would involve displacement of a water molecule.[9] The luminescence lifetime for Eu(THED)$^{3+}$ (20 μM) is identical within experimental error in the absence and presence of GpppG ($\tau^{-1}(H_2O) = 3.57$ ms^{-1}). Thus, it is likely that a Eu(THED)$^{3+}$−GpppG complex does not form to an appreciable extent under our experimental conditions.

Luminescence lifetime measurements were carried out to determine the number of water molecules bound to Eu(III) in the Eu(S-THP)−GpppG complex.[14,15] The difference in excited-state Eu(III) luminescence decay rates in H_2O and D_2O solutions is proportional to the number of water molecules (q)

(16) Frey, S. T.; Horrocks, W. D., Jr. *Inorg. Chim. Acta* **1995**, *229*, 383−390.

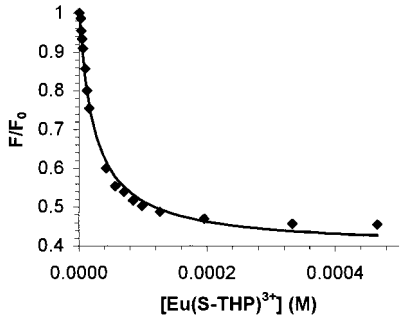


Figure 3. Emission intensity of m^{7}GpppG at 360 nm (λ_{ex} = 280 nm) as a function of the concentration of free Eu(S-THP)$^{3+}$ at 10 °C, pH 6.5, 50 mM buffer and with an initial m^{7}GpppG concentration of 11 μM. Data were fit to a 1:1 binding isotherm (eq 5)[11] to give $K = 4.4 \times 10^4$ M^{-1} (COD = 0.995).

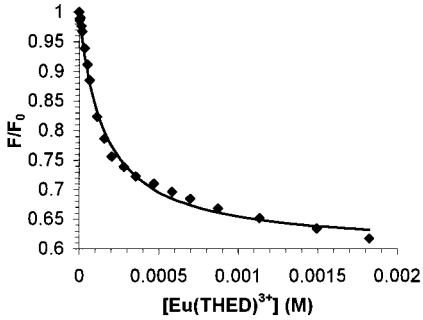


Figure 4. Emission intensity of m^{7}GpppG at 360 nm (λ_{ex} = 280 nm) as a function of the concentration of free Eu(THED)$^{3+}$ at 10 °C, pH 6.5, 50 mM buffer and with an initial m^{7}GpppG concentration of 9.7 μM. Data were fit to a 1:1 binding isotherm (eq 5)[11] to give $K = 5.9 \times 10^3$ M^{-1} (COD = 0.995).

bound to Eu(III).[14] The q value is determined by[15]

$$q = A(\tau^{-1}(H_2O) - \tau^{-1}(D_2O) - k_{XH}) \quad (3)$$

$$k_{XH} = \alpha + \beta n_{OH} \quad (4)$$

where α is the contribution from water molecules in the second coordination sphere and beyond,[15,17] n_{OH} is the number of alcohol O−H oscillators in the first coordination sphere of Eu(III), and τ^{-1} is the reciprocal excited-state lifetime in ms^{-1}. The value[15] of A is 1.11 waters × ms^{-1}, α is 0.30 ms^{-1}, and β is 0.45 ms^{-1}. By use of this equation, the number of bound water molecules (q) for Eu(S-THP)$^{3+}$ is 0.998 at pH 6.50 ($\tau^{-1}(H_2O)$ = 3.98 ms^{-1} and $\tau^{-1}(D_2O)$ = 0.981 ms^{-1}). For Eu(S-THP)−GpppG, q is −0.18 ($\tau^{-1}(H_2O)$ = 2.82 ms^{-1} and $\tau^{-1}(D_2O)$ = 0.883 ms^{-1}), consistent with a complex containing no bound water molecules. A negative value of q is, of course, impossible and may reflect the fact that the contribution from the second-sphere water molecules is somewhat variable.[18] In any case, the value of q is most consistent with a single bound water molecule in Eu(S-THP)$^{3+}$ being displaced upon binding GpppG.

Binding of Eu(S-THP)$^{3+}$ and Eu(THED)$^{3+}$ to m^{7}GpppG was measured by monitoring the quenching of fluorescence of m^{7}GpppG with added complex. Solutions contained 10 μM m^{7}GpppG, 40 mM buffer at either pH 6.5 or 7.5 and 10 °C. The normalized fluorescence (F/F_0) for m^{7}GpppG as a function of increasing concentrations of Eu(S-THP)$^{3+}$ or Eu(THED)$^{3+}$ is plotted in Figures 3 and 4, respectively. The data are fit to a 1:1 binding isotherm[11]

$$\frac{F}{F_0} = \frac{1 + CK[EuL^{3+}]_{free}}{1 + K[EuL^{3+}]_{free}} \quad (5)$$

C is defined as the ratio of fluorescence proportionality constants ($C = p_1/p_2$) of m^{7}GpppG with (p_1) and without (p_2) Eu(III) complex. (Fluorescence proportionality constants relate intensity to the concentration of species). Here, K is the formation constant for EuL^{3+} binding to m^{7}GpppG to form EuL^{3+}−GpppG (L = THED, THP). Because of the strong binding of Eu(S-THP)$^{3+}$ and the low ratios of complex to m^{7}GpppG used initially, it was necessary to take into account complex formation when determining the concentration of free Eu(S-THP)$^{3+}$. The concentration of free EuL^{3+} was calculated by iteration. Initial C and K values were obtained from a fit of eq 5 using total EuL^{3+} concentrations. The resulting values were used in

$$[EuL^{3+}]_{free} = \frac{-A + \sqrt{A^2 + 4K[EuL^{3+}]_{total}}}{2K} \quad (6)$$

to calculate corrected $[EuL^{3+}]_{free}$ values for each titration point ($A = K[GpppG]_{total} - K[EuL^{3+}]_{total} + 1$). The process was repeated until values for C and K converged. Fits to the data were excellent (COD = 0.995) and are good evidence that a 1:1 complex forms. Binding constants (K) at pH 6.5 are 5.9 × 10^3 M^{-1} and 4.4 × 10^4 M^{-1} for Eu(THED)$^{3+}$ and Eu(S-THP)$^{3+}$, respectively. At pH 7.5, Eu(S-THP)$^{3+}$ binds m^{7}GpppG with a binding constant of 1.5 × 10^5 M^{-1}. The larger binding constant at pH 7.5 is anticipated on the basis of the ionization properties of m^{7}GpppG.[19] The pK_a of the methylated guanosine of m^{7}GpppG is 7.35 at 20 °C. Deprotonation at N1 may cause m^{7}GpppG to bind more strongly to the cationic Eu(III) complex. No experiments were carried out with Eu(THED)$^{3+}$ at basic pH, since rapid cleavage of m^{7}GpppG is observed under these conditions.

Discussion

Previous work demonstrated that cleavage of GpppG by Eu(THED)$^{3+}$ is first order in Eu(THED)$^{3+}$ and GpppG over a range of Eu(III) complex concentrations.[5] If GpppG binding to Eu(THED)$^{3+}$ is in rapid preequilibrium prior to cleavage, a decrease in reaction order at higher concentrations of Eu(THED)$^{3+}$ would normally be observed. We had anticipated that GpppG would bind strongly to Eu(THED)$^{3+}$ given that GpppG is highly negatively charged and has three phosphate groups that normally bind well to metal ions.[7] However, studies here suggest that the binding constant for Eu(THED)$^{3+}$ to GpppG is less than 6 × 10^3, and this relatively small binding constant is consistent with the first-order reaction kinetics observed under the conditions of our experiments.[5] Nonetheless, it is possible that this type of complex (Eu(THED)−GpppG) is an important intermediate in cleavage. There is evidence that metal ion complexes with GpppG and nucleoside triphosphates are kinetically important in hydrolysis.[7,20,21] Metal ion catalyzed

(17) Dickins, R. S.; Parker, D.; de Sousa, A. S.; Williams, J. A. G. *Chem. Commun.* **1996**, 697−698.
(18) Beeby, A.; Clarkson, I. M.; Dickins, R. S.; Faulkner, S.; Parker, D.; Royle, L.; de Sousa, A. S.; Williams, J. A. G.; Woods, M. *J. Chem. Soc., Perkin Trans. 2* **1999**, 493−503.
(19) Wieczorek, Z.; Stepinski, J.; Jankowska, M.; Lonnberg, H. *J. Photochem. Photobiol. B* **1995**, *28*, 57−63.
(20) Sigel, H.; Hofstetter, F.; Martin, R. B.; Milburn, R. M.; Scheller-Krattiger, V.; Scheller, K. H. *J. Am. Chem. Soc.* **1984**, *106*, 7935−7946.
(21) Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107*, 3315−3321.

hydrolysis of phosphoric anhydrides such as ATP occurs through metal ion binding to a phosphate group followed by delivery of a nucleophilic metal hydroxide ligand.[20,21]

Eu(S-THP)$^{3+}$ binds m^{7}GpppG 7-fold more strongly than does Eu(THED)$^{3+}$. This is surprising considering that the solution properties of the two complexes are so similar.[8] The complexes have similar pK_a values for deprotonation of bound water or alcohol groups and nearly identical numbers of bound water molecules as a function of pH. Differences may arise due to specific interactions of functional groups on the two complexes with solvent or with GpppG. Eu(S-THP)$^{3+}$ binds to both GpppG and m^{7}GpppG with binding constants that are very similar for the two substrates. The relatively strong binding observed is surprising given that the Eu(S-THP)$^{3+}$ complex has but a single coordination site available for binding substrate if the macrocycle remains octadentate. Luminescence lifetime data are consistent with replacement of the bound water molecule in Eu(S-THP)$^{3+}$ to form Eu(S-THP)−GpppG, which contains an octadentate S-THP and bound GpppG.

Despite the fact that Eu(S-THP)$^{3+}$ binds to 5′-cap analogues, the complex does not promote cleavage of the 5′-cap of mRNA.[5] In the past we have observed that Eu(III) macrocyclic complexes that are not catalysts for phosphate diester transesterification or RNA cleavage do not even bind to substrate.[22] GpppG probably binds directly to Eu(S-THP)$^{3+}$ through one of the phosphate groups, and binding might be expected to activate GpppG to cleavage. However, Eu(S-THP)$^{3+}$ lacks a good nucleophile. The propanol pendent groups of Eu(S-THP)$^{3+}$ are poorer nucleophiles than the ethanol pendent groups of Eu(THED)$^{3+}$ or Eu(ATHC)$^{3+}$. Consistent with this reactivity trend, Eu(S-THP)$^{3+}$ cleaves activated phosphate diesters 56-fold more slowly than Eu(THED)$^{3+}$.[23]

Eu(ABHC)$^{3+}$ promotes cleavage of GpppG 2-fold more slowly than does Eu(THED)$^{3+}$. The relative magnitudes of these rate constants are consistent with the previously reported catalytic properties of Eu(III) complexes. Cleavage of bis(4-nitrophenyl)phosphate also occurs by attack of an alcohol pendent group of the Eu(III) complex; rate constants are similar for an Eu(III) macrocyclic complex containing one amide and three alcohol pendent groups compared to Eu(THED)$^{3+}$.[8] Thus, substitution of one alcohol group of Eu(THED)$^{3+}$ with an amide group does not substantially retard reactions where the pendent alcohol group is the nucleophile. This is consistent with the similar solution properties of Eu(THED)$^{3+}$ and Eu(III) macrocyclic complexes with mixed amide and alcohol pendent groups.[8] These Eu(III) complexes deprotonate to form an alkoxide or hydroxide complex; nearly identical pK_a values are observed for Eu(THED)$^{3+}$ compared to Eu(III) macrocyclic complexes containing one amide and three hydroxyethyl pendent groups.[8] In contrast, the hydrolytic cleavage of RNA by Eu(III) complexes is much more sensitive to macrocycle structure. Eu(III) macrocyclic complexes with mixed amide and alcohol pendent groups are 10- to 100-fold less effective catalysts for phosphate diester transesterification or RNA cleavage than Eu(THED)$^{3+}$.[8]

Eu(THED)$^{3+}$ and Zn(II) cooperatively promote cleavage of GpppG. A mechanism consistent with kinetic studies is shown in Scheme 1. The Eu(THED)$^{3+}$ complex provides the nucleophile[5,23] and may bind to a phosphate group of GpppG to activate it to nucleophilic attack. Zn(II) facilitates cleavage by acting as a second Lewis acid. Zn^{2+} may bind to and activate the phosphate group undergoing nucleophilic attack or, alternately, may bind to the GDP or GMP leaving group. Because metal ions are known to chelate to two or three different phosphate groups in a phosphoric anhydride,[24] it is also possible that Zn(II), which has several available coordination sites, may bind to and activate more than one phosphate group. That Mg(II) does not influence cleavage rates is consistent with its lower Lewis acidity compared to Zn(II). These studies suggest that dinuclear complexes with proper positioning of the two metal ions may be efficient catalysts for 5′-cap cleavage. Studies are underway to develop dinuclear metal ion complex catalysts.[6,7]

Acknowledgment. J.R.M. thanks the ACS Petroleum Research Fund, the National Institutes of Health, and the Alfred P. Sloan Foundation for support of this work. The Mark Diamond Research Fund of the Graduate Student Association of SUNY, Buffalo (award to D.M.E.) is acknowledged. Spectroscopic work at Pennsylvania State University was supported by NSF Grant CHE-9705788 to W.D.H. We thank Mark Panek for assistance and helpful discussions.

IC9912068

(22) Amin, S.; Voss, D. A., Jr.; Horrocks, W. D., Jr.; Lake, C. H.; Churchill, M. R.; Morrow, J. R. *Inorg. Chem.* **1995**, *34*, 3294−3300.

(23) Morrow, J. R.; Aures, K.; Epstein, D *J. Chem. Soc., Chem. Commun.* **1995**, 2431−2432.

(24) Orioli, P.; Cini, R.; Donati, D.; Mangani, S. *J. Am. Chem. Soc.* **1981**, *103*, 4446−4452.