Hydrolysis of a Model for the 5′-Cap of mRNA by Dinuclear Copper(II) and Zinc(II) Complexes. Rapid Hydrolysis by Four Copper(II) Ions

Kevin P. McCue and Janet R. Morrow*,†

Chemistry Department, State University of New York at Buffalo, Amherst, New York 14260-3000

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Two bis(triazacyclononane) ligands, 1,3-bis(1,4,7-triaza-1-cyclononyl)-*p*-xylene (*p*XTD) and 1,3-bis(1,4,7-triaza-1-cyclononyl)-*m*-xylene (*m*XTD), form stable dinuclear Cu(II) complexes (Cu_2L). At pH 7.3, the predominant species are bis(hydroxide) complexes ($Cu_2L(OH)_2^{2+}$) as determined by equilibrium modeling of pH−potentiometric measurements. Several dinuclear and mononuclear Cu(II) complexes and a dinuclear Zn(II) complex promote the hydrolysis of GpppG, a model for the 5′-cap of mRNA. At 0.125 mM complex, both Cu_2(*p*XTD) and Cu_2(*m*XTD) promote hydrolysis of GpppG approximately 100-fold more rapidly than does the monomeric Cu(TACN) complex (0.250 mM) at pH 7.3 and 37 °C (TACN = 1,4,7-triazacyclononane). The dependence of the rate constant on dinuclear Cu(II) complex concentration suggests that Cu_2(*p*XTD) promotes hydrolysis through both a 1:1 complex (Cu_2L−GpppG) and a 2:1 complex (($Cu_2L)_2$−GpppG). The 2:1 complex is 20-fold more reactive than the 1:1 complex; a first-order rate constant of 1.1×10^{-4} s^{-1} is determined for hydrolysis of the 2:1 complex. Cu_2(*m*XTD) effectively promotes the hydrolysis of GpppG only through a 2:1 complex which hydrolyzes with a first-order rate constant of 4.3×10^{-5} s^{-1}. Cu_2(*p*XTD) binds as a 1:1 complex to m^7GpppG with a binding constant of 27 000 M^{-1} as determined by use of fluorescence spectroscopy. Two Cu_2(*m*XTD) complexes bind stepwise to m^7GpppG with binding constants of 5300 and 12 000 M^{-1} for the first complex and second complex, respectively.

Introduction

Message RNAs synthesized by RNA polymerase II contain a structure which is referred to as the 5′-cap. The 5′-cap contains a N7-methylated guanosine and a triphosphate which is connected to the 5′-terminus of the mRNA. This structure plays a important role in RNA processing events including translation and stabilization of the mRNA. Destruction of the 5′-cap may inactivate a mRNA transcript, and this may be used as part of a strategy to selectively inhibit gene expression at the mRNA level.[1] Baker demonstrated that a Cu(II) phenanthroline complex promoted the hydrolysis of the 5′-cap of a mRNA transcript.[2] Cu(II) complexes hydrolyze the 5′-cap structure when the complexes are attached to an oligonucleotide which contains a sequence complementary to that of the mRNA (antisense oligonucleotide).[3] Inert lanthanide(III) complexes are even more effective at promoting cleavage of the 5′-cap both as free complexes and when tethered to oligonucleotides.[4,5] The 5′-cap cleavage reaction by Eu(III) complexes occurs through an alcohol group of the macrocyclic complex.[4] This results in a cross-linking reaction when the complexes are attached to antisense oligonucleotides.[5] Eu(III) macrocycle−oligonucleotide conjugates are more effective in mediating the selective inhibition of protein expression than the analogous unmodified antisense oligonucleotides. Encouraged by these results, we have initiated studies on dinuclear metal ion complexes in the interest of developing effective hydrolysis catalysts for the 5′-cap structure.

Many hydrolases in nature utilize two metal ions in catalyzing substitution reactions at phosphorus(V) substrates.[6,7] Biomimetic systems containing dinuclear Cu(II),[8−10] Zn(II),[11,12] Co(III),[13,14] or La(III)[15] complexes have been shown to enhance the rate of phosphate ester cleavage over that of mononuclear complexes. Nucleoside triphosphate hydrolysis has been shown to proceed more rapidly in the presence of two metal ions[16−18] as well. Our initial studies with lanthanide complexes[19] and Cu(II)

† Phone: (716) 645-6800 (2152). Fax: (716) 645-6963. E-mail: jmorrow@acsu.buffalo.edu.

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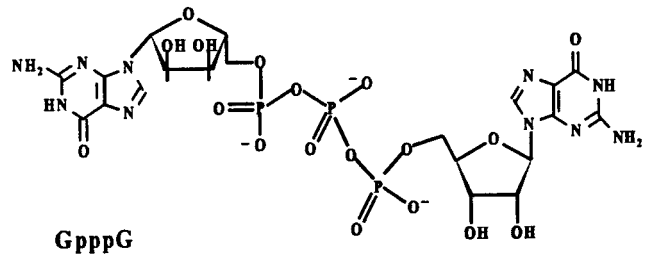
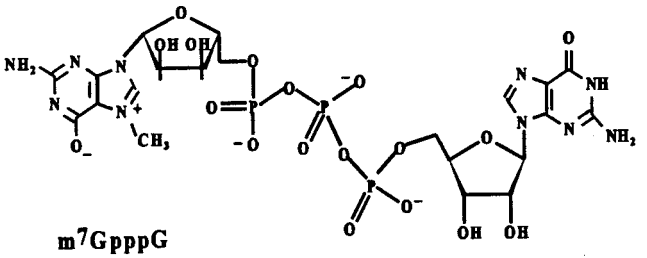
complexes[20] show that two metal ions effectively hydrolyze 5′-cap models. To study the two metal ion mechanism, we have examined dinuclear Cu(II) and Zn(II) complexes as hydrolysis catalysts for 5′-cap analogues, GpppG and m^{7}Gppp. Dinuclear



GpppG



m^{7}GpppG

ligands based on triazacyclononane were chosen since their Cu(II) complexes are very stable and their aqueous chemistry has been extensively studied.[21,22] In addition, mononuclear Cu(II) triazacyclononane complexes are good hydrolysis catalysts for phosphate esters.[23] This combination of well-defined aqueous chemistry and catalytic properties led us to examine these complexes as catalysts for 5′-cap hydrolysis.

Our previous work suggests that the mechanism of hydrolysis of GpppG by dinuclear Cu(II) complexes is dependent on the linker connecting the macrocyclic ligands.[20] In studies presented here, the kinetics of hydrolysis of a 5′-cap model substrate, GpppG, by two Cu(II) dinuclear complexes containing triazacyclononane macrocycles (Cu_2(mXTD) and Cu_2(pXTD); Figure 1) is studied in order to elucidate differences in reaction order of the two different Cu(II) complexes. Here Cu_2L designates all species present in a solution containing a 2:1 ratio of $Cu(NO_3)_2$ to ligand and CuL designates all species present for mononuclear complexes. pH−potentiometric measurements are used to determine metal−ligand binding constants and metal−water hydrolysis constants for these dinuclear Cu(II) complexes. The kinetics of hydrolysis of GpppG by two additional mononuclear Cu(II) complexes and one dinuclear Zn(II) complex are studied for comparison. The fluorescent properties of m^{7}GpppG are used to monitor binding of dinuclear Cu(II) complexes to 5′-cap analogues.

Experimental Section

Hepes buffer (*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid), Mes buffer (2-morpholinoethanesulfonic acid), Epps buffer (3-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), CHES buffer (2-(cyclohexylamino)ethanesulfonic acid), 1,3-dibromopropane, α,α′-dibromo-*m*-xylene, or α,α′-dibromo-*p*-xylene were of reagent grade and purchased from Sigma Chemicals or Aldrich. Solutions of $Cu(NO_3)_2$

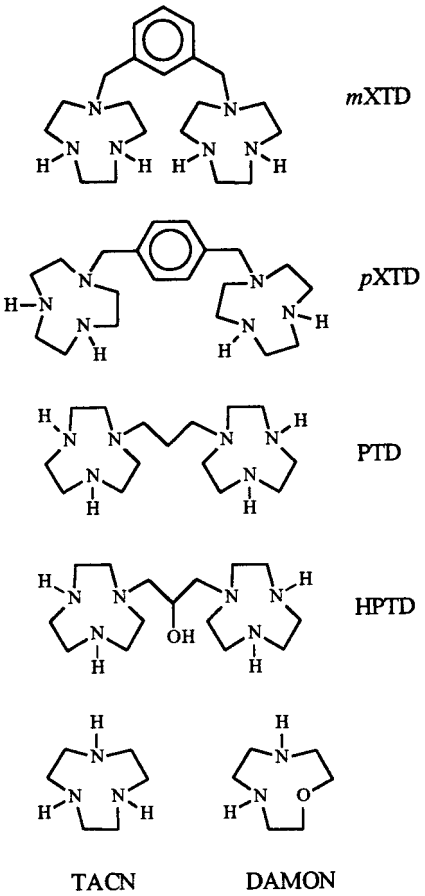


Figure 1. Ligands used in this study.

were titrated by using a literature method.[24] 1,4,7-Tris(*p*-tolylsulfonyl)-1,4,7-triazacyclononane, 1,4,7-triazacyclononane, and 1-oxa-4,7-triazacyclononane (DAMON) were synthesized according to a reported protocol.[25] GpppG and m^{7}GpppG were purchased from Pharmacia and the concentration determined by UV absorption at 250 nm (ϵ = 18 400 M^{-1} cm^{-1} for m^{7}GpppG or 19 100 M^{-1} cm^{-1} for GpppG). 1,3-Bis(1,4,7-triaza-1-cyclononyl)propane (PTD), 1,5-bis(1,4,7-triaza-1-cyclononyl)-*m*-xylene (*m*XTD), and 1,6-bis(1,4,7-triaza-1-cyclononyl)-*p*-xylene (*p*XTD) were prepared as described previously[20,22,26] and isolated as HBr salts. A Hewlett-Packard 5420 diode array UV−vis spectrophotometer equipped with a thermostated cell was used for UV−vis measurements.

pH−Potentiometric Titrations. All pH measurements were made with an Orion digital pH meter equipped with a temperature compensation probe. Titrations were carried out under a blanket of argon gas, and the solution temperature was maintained at 21 °C throughout the titration by use of a constant-tempertature bath. Solutions of ligand were prepared from the hydrobromide salts, and the concentration of ligand was determined by titrating the solutions with standardized NaOH. Solutions of $Cu(NO_3)_2$ were titrated as reported previously.[24] Solutions contained 0.100 M KNO_3 with a ligand concentration of 5.0 × 10^{-4} M. $Cu(NO_3)_2$ concentrations ranged from 5 × 10^{-4} to 1.0 × 10^{-3} M. Attainment of equilibrium was slow; data points were taken after the change in pH was less than 0.010 pH unit over 15 min.

Equilibrium Calculations. Analysis of the pH−potentiometric data was done using the computer programs *pKas* and *BESTA*.[27] Speciation

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Table 1. Acid Dissociation Constants for Protonated Forms of *m*XTD and *p*XTD at 21 °C in 0.10 M KNO_3[a]

reaction	log β *m*XTD	log β *p*XTD
$L + H^+ \leftrightarrow LH^+$	11.26(5)	11.18(6)
$L + 2H^+ \leftrightarrow H_2L^{2+}$	21.36(4)	21.27(2)
$L + 3H^+ \leftrightarrow H_3L^{3+}$	28.12(5)	27.78(4)
$L + 4H^+ \leftrightarrow H_4L^{4+}$	33.93(5)	33.6(1)

[a] Standard deviation in parentheses.

Table 2. Formation Constants for Cu(II) Complexes of *m*XTD and *p*XTD at 21 °C, in 0.10 M KNO_3[a]

reaction	log β *m*XTD	log β *p*XTD
$Cu^{2+} + L \leftrightarrow CuL^{2+}$	25.42(1)	22.97(3)
$Cu^{2+} + H^+ + L \leftrightarrow CuHL^{3+}$	27.25(2)	27.73(4)
$Cu^{2+} + 2H^+ L \leftrightarrow CuHL^{4+}$	33.06(4)	32.24(1)
$2Cu^{2+} + L \leftrightarrow Cu_2L^{4+}$	29.30(6)	29.78(2)
$2Cu^{2+} + L \leftrightarrow Cu_2LOH^{3+} + H^+$	23.6(1)	22.3(1)
$2Cu^{2+} + L \leftrightarrow Cu_2L(OH)_2^{2+}$	18.5(1)	16.1(1)

[a] Standard deviation in parentheses.

diagrams were created using the programs Spe and Spe plot. Statistical data for equilibrium modeling of the dinuclear Cu(II) complexes and for the ligands alone were within acceptable values.

Fluorescence Studies. Fluorescence spectra were measured using a SLM-aminco model 8100 spectrofluorimeter with a MC200 monochromator used for emission and MC400 monochromator for excitation. The samples were placed in a constant-temperture cell holder, and a Brinkman Lauda water bath was used to control the temperature. The excitation wavelength was 287 nm, and the emission was recorded at 358 nm. Experiments were run at least in triplicate; solutions contained m^7GpppG (10 μM) and 40 mM buffer for all fluorimetry experiments. Standard deviations for binding constants were 10% or less. No hydrolysis was observed under the conditions of the fluorescence experiments as confirmed by HPLC analysis of m^7GpppG solutions under similar conditions. Solutions for the Job plot experiments (method of continuous variations) were prepared with the restriction that $[Cu_2(L)] + [m^7GpppG] = 14$ μM and χ (mole fraction) values of the two components were varied.

Kinetic Data. Rate constants were determined by monitoring the disappearance of GpppG by use of a Waters 600E HPLC equipped with a 490 UV−vis detector. The dinucleotide ApU was used as an internal standard. Reaction solutions were analyzed on a C18 column (250 mm × 4.6 mm) and eluted with a 50:50 mixture of solvents A and B. Solvent A contained 50 mM KH_2PO_4 and 5.0 mM *tert*-butylammonium phosphate at pH 5.0, and solvent B contained 5.0 mM *tert*-butylammonium phosphate in a 50:50 water−methanol mixture.[4] Reaction solutions were incubated at 37 °C and contained 30 μM GpppG and 40 mM Hepes buffer at pH 7.3. Hydrolysis of GpppG in the presence of a 10-fold excess of dinuclear Cu(II) and Zn(II) complex was first order in GpppG; data plotted as $-\ln(A/A_0)$ vs time were linear for greater than 3 half-lives. Reaction rate constants were determined by averaging data from three to seven experiments. Standard deviations for rate constants were 10% or less except for mononuclear complexes which hydrolyzed GpppG very slowly where the standard deviation was as large as 20%.

Results

Data from pH−potentiometric titrations of ligand and solutions containing different ratios of $Cu(NO_3)_2$ to ligand were fit using the computer programs *pKas* and *Besta*.[27] log β and log *K* values tabulated for Cu(II) complexes and ligands in Tables 1−3 are similar to those reported previously for dinuclear Cu(II) complexes of triazacyclononane containing alkyl chain linkers.[21,22] Species distribution diagrams for Cu_2(*m*XTD) and Cu_2(*p*XTD) (Supporting Information Figures 1 and 2) were created from the equilibrium model and log β values. At pH 7.3 where kinetic studies were carried out, the $Cu_2(L)(OH)_2^{2+}$ species is the major species for Cu_2(*m*XTD) (>95%) and for Cu_2(*p*XTD) (80%).

Table 3. Equilibrium Constants for the Hydrolysis of Cu(II) Complexes of *m*XTD and *p*XTD at 21 °C in 0.10 M KNO_3

reaction	log K_a *m*XTD	log K_a *p*XTD
$K_a(Cu_2L)$ $Cu_2L^{4+} \leftrightarrow Cu_2LOH^{3+} + H^+$	−5.8	−7.5
$K_a(Cu_2LOH)$ $Cu_2LOH^{3+} \leftrightarrow Cu_2L(OH)_2^{2+} + H^+$	−5.1	−6.2

Binding of Cu_2(*m*XTD) and Cu_2(*p*XTD) to m^7GpppG was examined by monitoring changes in the fluorescence emission intensity of the substrate upon addition of Cu(II) complex. Conditions for fluorescence spectroscopy experiments were chosen so that no hydrolysis of m^7GpppG was observed over the course of the experiments (10 °C and pH 7.3). The method of continuous variations was used to examine the stoichiometry of dinuclear Cu(II) complex binding to m^7GpppG at 10 °C and pH 7.3. The Job's plot for Cu_2(*p*XTD) binding has a sharp intersection at $\chi = 0.5$ suggesting that, under these conditions, the 1:1 Cu_2(*p*XTD)−m^7GpppG complex predominates (Supporting Information Figure 3). Cu_2(*m*XTD) binds m^7GpppG more weakly than doesCu_2(*p*XTD), and the Job's plot for this system failed to provide information about the stoichiometry of the complex with m^7GpppG.

Shown in Figure 2 is a plot of the fluorescence emission intensity as a function of Cu(TACN), Cu_2(*p*XTD), and Cu_2(*m*XTD) concentration. Data for Cu_2(*p*XTD) is fit to a 1:1 binding isotherm as given in eq 2 where I_n/I_o is the ratio of

$$Cu_2L + m^7GpppG \overset{K_1}{\rightleftharpoons} GpppG{-}Cu_2L \quad (1)$$

$$I_n/I_o = \frac{1 + (f_1/f_s)K_1[Cu_2L]}{1 + K_1[Cu_2L]} \quad (2)$$

$$m^7GpppG{-}Cu_2L + Cu_2L \overset{K_1}{\rightleftharpoons} m^7GpppG{-}(Cu_2L)_2 \quad (3)$$

$$I_n/I_o = \frac{1 + (f_1/f_s)K_1[Cu_2L] + (f_2/f_s)K_1K_2[Cu_2L]^2}{1 + K_1[Cu_2L] + K_1K_2[Cu_2L]^2} \quad (4)$$

fluorescence intensity with and without added Cu(II) complex, f_1 and f_s are the fluorescence proportionality constants of bound m^7GpppG and free GpppG, respectively, *M* is the molarity of metal complex, and K_1 is the binding constant. A binding constant of 27 000 M^{-1} with an f_1/f_s of 0.070 and a coefficient of determination (COD) of 0.999 was determined. For Cu(TACN) only a weak linear decrease in fluorescence excitation was observed and a binding constant could not be obtained. Data for Cu_2(*m*XTD) binding to m^7GpppG were fit to an expression which included stepwise binding of two Cu(II) dinuclear complexes (eqs 3 and 4). In eq 4, f_1 and f_2 are fluorescence proportionality constants for the 1:1 complex and the 2:1 complex, respectively. Note that the lower the value of f_1/f_s or f_2/f_s, the more efficient the quenching and the lower the intensity of fluorescence. The value of f_2/f_s is obtained from the limiting fluorescence emission intensity where the 2:1 species predominates. In addition, f_2/f_s is less than f_1/f_s since quenching by two Cu(II) complexes is likely more effective than quenching by one complex. With these assumptions, f_2/f_s was fixed at 0.10, f_1/f_s was changed in increments of 0.05 from 0.10 to 0.50, and

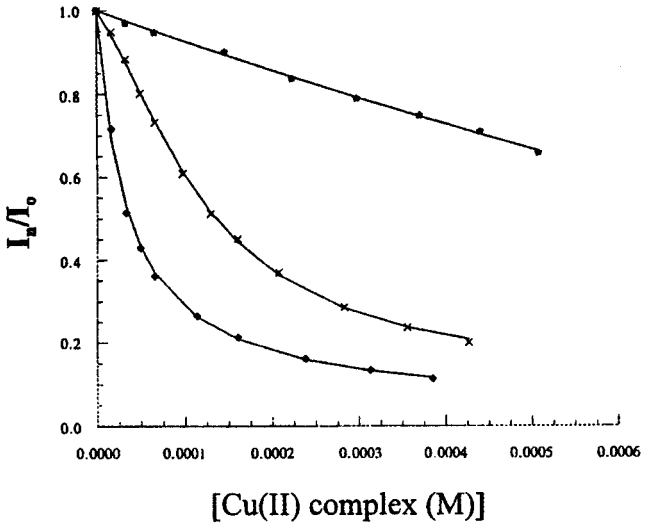

Figure 2. Quenching of fluorescence emission intensity at 358 nm (excitation at 287 nm) of m^7GpppG upon addition of Cu(II) complexes: ★ = Cu(TACN); × = Cu_2(*m*XTD); ◆ = Cu_2(*p*XTD). Solutions were maintained at 10 °C, pH 7.3, and 40 mM Hepes with 10 μM m^7GpppG. I_n/I_o is the ratio of fluorescence intensity with and without added Cu(II) complex. Data for Cu_2(*m*XTD) are fit to eq 4, and data for Cu_2(*p*XTD) are fit to eq 2 as described in the text.

Table 4. Pseudo-First-Order Rate Constants for the Hydrolysis of GpppG by Cu(II) and Zn(II) Complexes at 37 °C and pH 7.3[a]

complexes	10^5k_o (s^{-1})	complexes	10^5k_o (s^{-1})
Cu_2(*m*XTD) (125 μM)	3.6(0.4)	Cu_2(PTD)	1.7(0.2)
Cu_2(*p*XTD) (125 μM)	2.2(0.2)	Cu_2(HPTD)	1.8(0.2)
Cu(TACN) (250 μM)	0.023(0.08)	Zn_2(HPTD)	0.53(0.05)
Cu_2(*m*XTD)	3.9(0.2)	Cu(DAMON)	0.16(0.04)
Cu_2(*p*XTD)	5.6(0.4)	Cu(TACN)	0.12(0.02)

[a] Reactions were in 40 mM Hepes buffer with 0.500 mM complex for dinuclear species or 1.00 mM complex for mononuclear species unless otherwise noted. Standard deviations are in parentheses.

K_1 and K_2 were allowed to vary. Fitting of the data to (4) gives an f_1/f_s ratio of 0.38, K_1 of 5300, and K_2 of 12000 (COD = 0.999, MSC = 7.64).[28] Note that MSC (model selection criterion) values are an appropriate goodness-of-fit statistic when comparing models with different numbers of variables;[28] the larger the MSC, the better the fit to the model. Other possibilities include simultaneous binding of a tetranuclear Cu(II) complex to m^7GpppG or binding of a single Cu_2(*m*XTD) to m^7GpppG (eq 2). Fits to either of these models gave much poorer goodness-of-fit statistics (Supporting Information Figure 4).

Hydrolysis of GpppG by mononuclear and dinuclear metal ion complexes was examined. Disappearance of GpppG was monitored by use of an HPLC assay,[4] and the sole products detected were GMP and GDP as determined by co-injection with authentic standards. Pseudo-first-order rate constants for mononuclear and dinuclear Cu(II) complexes and a dinuclear Zn(II) complex are listed in Table 4 for complexes at pH 7.3 and 37 °C, with 40 mM HEPES buffer. All dinuclear Cu(II) complexes hydrolyzed GpppG more rapidly than the mononuclear Cu(TACN). At the lower concentrations given in Table 4, hydrolysis of GpppG by dinuclear Cu(II) complexes of *m*XTD and *p*XTD is approximately 100-fold more rapid per Cu(II) ion than it is for the monomeric Cu(TACN) complex under similar conditions. The Cu(II) and Zn(II) dinuclear complexes of HPTD were less effective than the Cu(II) complexes containing xylyl linkers. Hydrolysis of GpppG by Cu(DAMON) or by Cu(TACN) had similar rate constants. In the absence of catalyst, only 3% of the GpppG was hydrolyzed over a period of 5 days.

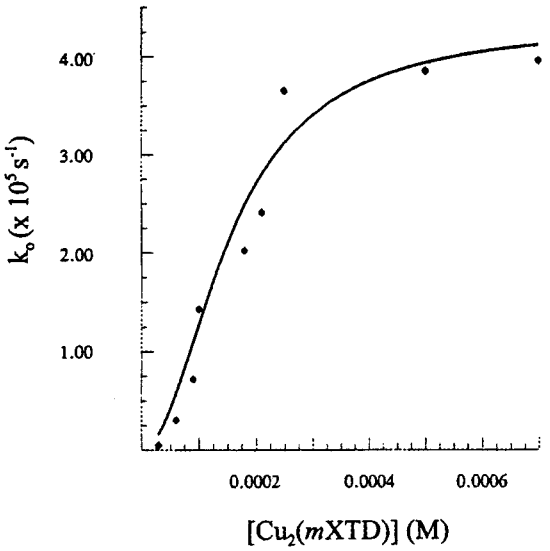

Figure 3. Dependence of pseudo-first-order rate constant on the concentration of Cu_2(*m*XTD) at 37 °C, pH 7.3, 40 mM Hepes, and 30 μM GpppG.

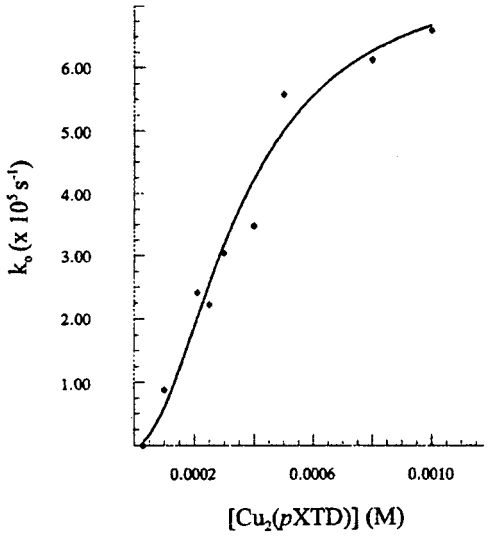

Figure 4. Dependence of pseudo-first-order rate constant on the concentration of Cu_2(*p*XTD) at 37 °C, pH 7.3, 40 mM Hepes, and 30 μM GpppG.

Further kinetic studies were conducted to study the mechanism of hydrolysis of GpppG by two of the more promising dinuclear complexes. We previously[20] reported that hydrolysis of GpppG by Cu_2(*p*XTD) is first order in complex in the concentration range 0.100−0.500 mM with an apparent second-order rate constant of 0.10 M^{-1} s^{-1}. Hydrolysis by Cu_2(*m*XTD) was second order in complex for concentrations ranging from 0.0300 mM to 0.210 mM with an apparent third-order rate constant of 730 M^{-2} s^{-1}. The dependence of GpppG hydrolysis on concentration of Cu_2(*p*XTD) and Cu_2(*m*XTD) was studied further (Figures 3 and 4). Both complexes exhibited saturation kinetics at higher concentrations of complex.

Kinetic data for hydrolysis by Cu_2(*m*XTD) are modeled by assuming that a 2:1 complex forms (eqs 5−7), consistent with m^7GpppG binding stoichiometry and with previous kinetic data.[20] In eq 7, k_o is the observed rate constant and k_p and k_q are first-order rate constants for the hydrolysis of the 1:1 and 2:1 Cu_2L−GpppG complexes, respectively. A steep slope is

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$$\mathrm{GpppG} + \mathrm{Cu_2L} \overset{K_{m1}}{\rightleftharpoons} \mathrm{GpppG{-}Cu_2L} \xrightarrow{k_p} \mathrm{P} \qquad (5)$$

$$\mathrm{GpppG{-}Cu_2L} \overset{K_{m2}}{\rightleftharpoons} \mathrm{GpppG{-}(Cu_2L)_2} \xrightarrow{k_q} \mathrm{P} \qquad (6)$$

$$k_o = \frac{k_p K_{m1}[\mathrm{Cu_2L}] + k_q K_{m1} K_{m2}[\mathrm{Cu_2L}]^2}{1 + K_{m1}[\mathrm{Cu_2L}] + K_{m1}K_{m2}[\mathrm{Cu_2L}]^2} \qquad (7)$$

observed with an abrupt leveling of the rate constant at about 0.25 mM, consistent with cooperative binding where $K_{m2} > K_{m1}$.[29] The rate constant k_p must be much less than k_q since a second-order dependence on metal ion complex is observed for the concentration range prior to saturation. If k_p is zero, the data are fit to (7), and k_q is 4.3×10^{-5} s^{-1}, K_{m1} is 400, and K_{m2} is 110 000 (COD = 0.974, MSC = 2.63). It is possible that k_p is not zero; however, the small magnitude of the first binding constant (K_{m1}) makes it impossible to determine k_p accurately. The data in Figure 3 also fit to a model with two dinuclear complexes forming a tetranuclear complex prior to binding to GpppG (eqs 8−10). In this case the product of K_d and K_{m3} is

$$2\mathrm{Cu_2L} \overset{K_d}{\rightleftharpoons} (\mathrm{Cu_2L})_2 \qquad (8)$$

$$(\mathrm{Cu_2L})_2 + \mathrm{GpppG} \xrightarrow{K_{m3}} (\mathrm{Cu_2L})_2\mathrm{{-}GpppG} \xrightarrow{k_q} \mathrm{P} \qquad (9)$$

$$k_0 = \frac{k_q K_d K_{m3}[\mathrm{Cu_2L}]^2[\mathrm{GpppG}]}{1 + K_d K_{m3}[\mathrm{Cu_2L}]^2} \qquad (10)$$

4.2×10^7 and k_q remains 4.3×10^{-5} s^{-1} (COD = 0.946, MSC = 2.64). Thus the only constant which can be determined reliably from this analysis is k_q, the rate constant for hydrolysis of the 2:1 complex.

The dependence of GpppG hydrolysis on the concentration of Cu$_2$(*p*XTD) (Figure 4) is characterized by a first-order dependence in the low concentration range followed by a decrease in slope without complete kinetic saturation at higher concentrations. Due to this feature at high concentrations of complex, the data did not fit to a kinetic equation for preequilibrium binding of a single Cu(II) dinuclear complex to GpppG. Equation 7 which includes terms for binding to and hydrolysis of GpppG by a second dinuclear Cu(II) complex fit the data much better. Fitting to eq 7 with the assumption that K_{m1} is greater than K_{m2} gave $k_p = 6 \times 10^{-6}$ s^{-1}, $k_q = 1.1 \times 10^{-4}$ s^{-1}, $K_{m1} = 7300$, and $K_{m2} = 1500$ (COD = 0.982).

Discussion

Both *m*XTD and *p*XTD strongly bind two Cu(II) ions. The predominant species at a 2:1 ratio of Cu(NO$_3$)$_2$ to ligand at pH 5 is a dinuclear complex (Cu$_2$(L)$^{4+}$) while at neutral pH the predominant species is a bis(hydroxide) dinuclear Cu(II) complex (Cu$_2$L(OH)$_2^{2+}$). Both *m*XTD and *p*XTD also form extremely stable 1:1 (CuL) complexes (log β 25.42 and 22.97). Cu(*m*XTD) is more stable than Cu(*p*XTD) by over 2 orders of magnitude. The consequence of this difference in stability is a difference in speciation at low pH values for the two complexes. At pH 4.5, Cu(NO$_3$)$_2$ solutions with *m*XTD in a 2:1 ratio contain 30% LM and 60% LM$_2$ complexes, whereas, for *p*XTD, the LM$_2$ species is nearly 100%. The difference in stability between ML complexes is reminiscent of that observed by Zompa[21,22] and co-workers for dinuclear Cu(II) complexes of triazacyclononane connected by alkyl linkers. They proposed that the enhanced stability of a Cu(II) complex with a propyl linker (Cu(PTD)) was due to formation of a species with one Cu^{2+} ion bound to both triazacyclononane rings in the same ligand (intramolecular bis-complex). Longer linkers favor coordination of two triazacyclononane rings from different ligands to give an intermolecular bis-complex. Formation constant values here suggest that Cu(*m*XTD) may form the stable intramolecular bis-complex. Modeling studies suggest that the *p*-xylyl linker prevents the two Cu(II) from forming the intramolecular bis-complex; however, we cannot rule out the formation of an intermolecular bis-complex.

The hydrolysis constants for Cu$_2$(*p*XTD) and Cu$_2$(*m*XTD) are similar to those of Cu$_2$(PTD).[25,26] For all three complexes, the K_a of Cu$_2$L^{4+} to form Cu$_2$L(OH)$^{3+}$ is smaller than the K_a of Cu$_2$L(OH)$^{3+}$ to form Cu$_2$L(OH)$_2^{2+}$. This indicates that Cu$_2$L-(OH)$_2^{2+}$ is more stable than Cu$_2$L(OH)$^{3+}$. In addition, both pK_a values of the metal-bound waters for the Cu(II) dinuclear complex of *m*XTD are nearly one pH unit lower than those of the dinuclear complex of *p*XTD. We attribute this discrepancy to the formation of different types of hydroxide complexes. Cu$_2$-(*m*XTD)(OH)$_2^{2+}$ contains two hydroxide ligands which bridge in an intramolecular sense for the complex in the solid state.[30] Molecular mechanics calculations confirm that the two Cu(II) centers in Cu$_2$(*m*XTD) support μ-hydroxy bridges without increasing the strain energy of the ligand.[20,30] In contrast, modeling studies aided by molecular mechanics calculations suggest that intramolecular hydroxide bridges cannot form between the two Cu(II) centers in Cu$_2$(*p*XTD) without a large increase in ligand strain energy.[20] Previous studies have shown that the deprotonation of water ligands to form μ-hydroxide ligands leads to pK_a values that are lower than those of similar water ligands that cannot form intramolecular hydroxide bridges upon deprotonation.[31,32] While Cu$_2$(*p*XTD)(OH)$_2^{2+}$ cannot contain intramolecular bridging hydroxides, it is possible that complexes containing intermolecular bridging hydroxides may be present in solution. If intermolecular bridging hydroxide complexes are present in significant amounts, there should be a dependence of the pK_a values for Cu(II) water ligands on concentration of dinuclear complex. Unfortunately, titrations at higher concentrations of complex (1.00 mM *p*XTD, 2.00 mM Cu(NO$_3$)$_2$) lead to the formation of precipitates at neutral pH values, and this limited our investigation of the formation of higher order complexes.

Binding of the two dinuclear complexes Cu$_2$(*m*XTD) and Cu$_2$(*p*XTD) to m^{7}GpppG was studied by monitoring m^{7}GpppG fluorescence quenching in the presence of the Cu(II) complexes.[36−38] The fluorescent properties of m^{7}GpppG have been utilized previously to examine its solution chemistry[33−35] including the effect of protons and metal cations such as Mg(II) on cap structure. The purpose of these studies was to gain information about the stoichiometry and strength of binding of dinuclear complexes to the 5′-cap. However the expectation was

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that binding constants obtained from fluorescence data would differ from those obtained from kinetic data given that a different cap model is used for hydrolysis kinetics (GpppG) than for binding studies (m^7GpppG) and different temperatures were used in order to inhibit cleavage during binding studies.

Both dinuclear complexes bind to m^7GpppG more strongly than does the Cu(TACN) complex, a result which was anticipated on the basis of charge considerations. Binding constants and stoichiometry of binding differ for the two dinuclear complexes. The most reasonable explanation for this lies in the different structures of the two bis(hydroxide) Cu(II) complexes. The Cu_2(*p*XTD)$(OH)_2^{2+}$ complex contains no intramolecular bridging hydroxides and should readily bind to m^7GpppG by displacement of water ligands. Molecular mechanics calculations suggest that Cu_2(*p*XTD)$(OH)_2^{2+}$ may bind to GpppG through two different phosphate groups; binding of a bridging phosphate group is not possible.[20] Cu_2(*p*XTD) forms a stable 1:1 complex with m^7GpppG as determined by analysis of a Job's plot and fitting of the binding isotherm. It is possible that a second Cu_2(*p*XTD) binds to m^7GpppG, but it must bind more weakly than the first. In contrast, Cu_2(*m*XTD)$(OH)_2^{2+}$ contains intramolecular hydroxide bridges in the solid state and probably in solution.[30] To bind to m^7GpppG, one of the μ-hydroxide ligands must be lost if the Cu(II) centers are to remain 5-coordinate. Binding of Cu_2(*m*XTD)$^{4+}$ to m^7GpppG thus would compete with formation of the bis(μ-hydroxide) species. This would lower the effective binding constant for GpppG under conditions where formation of the bis(μ-hydroxide) complex is favorable. Consistent with this hypothesis, the first binding constant for Cu_2(*m*XTD) to m^7GpppG is lower than that of Cu_2(*p*XTD). Curiously, binding of a second Cu_2(*m*XTD) is stronger than binding of the first complex. One model for this cooperative binding involves facilitation of a conformational change of GpppG by the first dinuclear complex which induces the second dinuclear species to bind more strongly. Alternately, there may be an interaction between the two bound Cu(II) dinuclear complexes, perhaps through formation of an intermolecular bridging hydroxide. In this model, the negatively charged GpppG catalyzes the interaction of two Cu_2(*m*XTD) molecules. Binding data obtained by fluorescence spectroscopy are not fit satisfactorily by a model whereby the two Cu_2(*m*XTD) complexes dimerize prior to binding to m^7GpppG (Supporting Information Figure 4), suggesting that binding is stepwise.

Two different Cu(II) complexes of tridentate macrocyclic ligands promote GpppG hydrolysis. The pseudo-first-order rate constant for Cu(DAMON) is similar to that of the Cu(TACN) complex. The pK_a values for Cu(II) water ligands in the complexes (7.2 for Cu(TACN) and 7.3 for Cu(DAMON))[39] are close, suggesting that these complexes have similar Lewis acidity and might be expected to have similar catalytic activity. It is interesting to note that pseudo-first-order rate constants for these monomeric macrocyclic complexes are approximately 10-fold lower than that of Cu(II)-promoted hydrolysis of GpppG in the presence of phenanthroline under similar conditions.[2] There are numerous factors that are important in hydrolysis catalyst efficiency other than Lewis acidity. For example, the extent formation of dinuclear complexes which are inactive as catalysts appears to be very imporant.[23,40] In any case, the large stability constants of macrocyclic complexes facilitate mechanistic studies compared to more weakly binding ligands such as phenanthroline.

All dinuclear Cu(II) complexes have larger apparent pseudo-first-order rate constants than any of the mononuclear Cu(II) complexes under similar conditions. Only relatively small differences in rate constants are observed for the four different dinuclear Cu(II) complexes. On the basis of similar solution pK_a values[21,22] and modeling studies, Cu_2(PTD)$(OH)_2^{2+}$ is likely to have two intramolecularly bridging hydroxide ligands and might be expected to have catalytic properties similar to those of Cu_2(*m*XTD). Addition of an alcohol group which may bridge two Cu(II) ions in the dinuclear ligand HPTD does not lead to a better Cu(II) catalyst.

The Zn_2(HPTD) complex is surprisingly reactive in comparison to the Cu(II) dinuclear complexes. Zn(TACN) complexes are generally not as effective as Cu(TACN) complexes as hydrolysis catalysts[41] and we anticipated that the dinuclear complexes would be less active as well. Our pH−potentiometric data[42] suggest that in solutions with a 2:1 ratio of $Zn(NO_3)_2$ to HPTD at pH 7.3, the Zn(II) complexes (Zn_2(HPTD)$^{4+}$) do not contain hydroxide ligands. Since the Zn(II) hydroxide complex is not present in substantial concentrations at pH 7.3 and a metal ion hydroxide complex is likely to be the catalytically active species (see below), neutral pH is probably not optimal for these complexes as catalysts.

Both dinuclear Cu(II) complexes accelerate GpppG hydrolysis through formation of a 2:1 complex ($(Cu_2L)_2$−GpppG). For Cu_2(*p*XTD), the 2:1 complex hydrolyzes 20-fold more rapidly than does the 1:1 complex. Rate constants for the hydrolysis of GpppG in the 2:1 complex (k_q) differ little for the two complexes; k_q is 2.5-fold higher for Cu_2(*p*XTD) than for Cu_2(*m*XTD). It is the differences in the binding constants that gives rise to the very different shapes of the kinetic saturation curves for the two dinuclear complexes. Our original interpretation[20] of the data from subsaturating concentrations of metal ion complex was that Cu_2(*m*XTD) hydrolyzed GpppG through a 2:1 complex whereas Cu_2(*p*XTD) hydrolyzed GpppG through a 1:1 complex. The kinetic data presented here suggest that both complexes hydrolyze GpppG more efficiently through a 2:1 complex. Both kinetic data and fluorescence binding data suggest that the second Cu_2(*p*XTD) binds more weakly than does the first. In contrast, both kinetic data and fluorescence binding data suggest that a second Cu_2(*m*XTD) binds more strongly than does the first. From the kinetic data, we cannot distinquish between stepwise binding of the two Cu_2(*m*XTD) and simultaneous binding of two Cu_2(*m*XTD). However, the fluorescence binding data suggest that stepwise binding occurs, and we favor a similar model for Cu_2(*m*XTD) binding to GpppG.

A mechanism consistent with kinetic data for Cu_2(*p*XTD) involves binding of a dinuclear metal ion complex to GpppG followed by delivery of a Cu(II)-bound nucleophile to promote hydrolysis. In analogy to the mechanism of hydrolysis of phosphate esters[43] and phosphoric anhydrides,[16−18] the nucleophilic species here is likely to be a metal ion bound hydroxide. Active catalysts for phosphate ester hydrolysis contain a site for binding the ester and an hydroxide nucleophile in a cis orientation. For phosphate diester hydrolysis, comparison of the

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kinetic pH rate profile and pH−potentiometric studies of the complex in solution has lent support to the hypothesis that a metal ion hydroxide is the nucleophile.[43] In contrast to phosphate diesters, GpppG is a highly charged ligand which modifies the Lewis acidity of the metal ion center and the pK_a value of the metal ion water ligands upon binding. Kinetic pH rate profiles for metal ion promoted hydrolysis of GpppG do not correlate with pK_a values of the free metal ion complexes since binding of GpppG suppresses deprotonation of the metal ion bound water.[19] A similar observation has been made for metal ion bound nucleoside triphosphate complexes. For nucleoside triphosphate hydrolysis, kinetic rate constants are compared to species distribution diagrams for metal ion complex and substrate to unravel the mechanism of hydrolysis.[16−18] Unfortunately, pH−potentiometric studies of metal ion complexes in the presence of GpppG could not be carried out here as large quantities of GpppG are very expensive.

Dinuclear metal ion sites may hydrolyze phosphate esters and RNA by double Lewis acid activation with one metal ion binding to the incoming nucleophile, the second metal ion binding to the leaving group, and both metal ions binding to the phosphate diester.[44] Hydrolysis of GpppG by two metal ions in a 1:1 complex probably requires interaction of two metal ions at two different phosphate groups similar to the mechanism proposed for nucleoside triphosphate hydrolysis by two metal ions.[16−18] In this scheme, one metal ion delivers the nucleophile and the second metal ion binds to the GDP leaving group through one or both phosphates. Cu_2(*p*XTD) may promote hydrolysis through such a pathway since hydrolysis occurs through a 1:1 complex. For both dinuclear Cu(II) complexes, however, rapid hydrolysis occurs in a 2:1 complex; thus four metal ions are involved. One reason that two dinuclear complexes are involved may be that the two Cu(II) centers in the dinuclear complexes are not oriented optimally for binding both to the leaving group and the phosphate ester undergoing attack. Binding of a second dinuclear complex then would further accelerate hydrolysis. The focus of future studies will be to design multinuclear metal ion complexes that hydrolyze GpppG through a 1:1 complex in order to facilitate studies with metal ion macrocycle−oligonucleotide conjugates.

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Supporting Information Available: Figures showing speciation plots, Job's plots, and fluorescence quenching data. This material is available free of charge via the Internet at http://pubs.acs.org.

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