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Article

Kinetics and Mechanism of Hydrolysis of a Model Phosphate Diester by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ (Me$_3$tacn = 1,4,7-Trimethyl-1,4,7-triazacyclononane)

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The kinetics of hydrolysis of bis(*p*-nitrophenyl)phosphate (BNPP) by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ has been studied by spectrophotometrical monitoring of the release of the *p*-nitrophenylate ion from BNPP. The reaction was followed for up to 8000 min at constant BNPP concentration (15 μM) and ionic strength (0.15 M) and variable concentration of complex (1.0–7.5 mM) and temperature (42.5–65.0 °C). Biphasic kinetic traces were observed, indicating that the complex promotes the cleavage of BNPP to NPP [(*p*-nitrophenyl)phosphate] and then cleavage of the latter to phosphate, the two processes differing in rate by 50–100-fold. Analysis of the more amenable cleavage of BNPP revealed that the rate of BNPP cleavage is among the highest measured for mononuclear copper(II) complexes and is slightly higher than that reported for the close analogue [Cu(*i*Pr$_3$tacn)(OH$_2$)$_2$]$^{2+}$. Detailed analysis required the determination of the pK_a for [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ and the constant for the dimerization of the conjugate base to [(Me$_3$tacn)Cu(OH)$_2$Cu(Me$_3$tacn)]$^{2+}$ (K_{dim}). Thermodynamic parameters derived from spectrophotometric pH titration and the analysis of the kinetic data were in reasonable agreement. Second-order rate constants for cleavage of BNPP by [Cu(Me$_3$tacn)(OH$_2$)(OH)]$^+$ and associated activation parameters were obtained from initial rate analysis ($k = 0.065$ M^{-1} s^{-1} at 50.0 °C, $\Delta H^{\ddagger} = 56 \pm 6$ kJ mol^{-1}, $\Delta S^{\ddagger} = -95 \pm 18$ J K^{-1} mol^{-1}) and biphasic kinetic analysis ($k = 0.14$ M^{-1} s^{-1} at 50.0 °C, $\Delta H^{\ddagger} = 55 \pm 6$ kJ mol^{-1}, $\Delta S^{\ddagger} = -92 \pm 20$ J K^{-1} mol^{-1}). The negative entropy of activation is consistent with a concerted mechanism with considerable associative character. The complex was found to catalyze the cleavage of BNPP with turnover rates of up to 1 per day. Although these turnover rates can be considered low from an application point of view, the ability of the complexes to catalyze phosphate ester cleavage is clearly demonstrated.

Introduction

Metal-ion-promoted hydrolysis of phosphate esters is an extremely active area of research.[1–16] This interest is derived

from the facts that the backbones of DNA and RNA both contain phosphodiester bonds and that numerous other phosphate esters play vital biological roles. Phosphate ester bonds are extremely resistant to hydrolysis (for example, the half-life for cleavage has been estimated to be greater than 100000 years for DNA at neutral pH and 25 °C[7,17] and 4 years for RNA[7] in the absence of a catalyst), and nature has developed an impressive array of metalloenzymes, often

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with two or more metal ions at their active sites, that catalyze the hydrolytic cleavage of such bonds and achieve turnover rates that are sufficient to sustain life. Interest in providing an understanding of the mechanisms by which such reactions occur has stimulated the development of small-molecule mimics that catalyze these hydrolytic cleavage reactions. Further impetus has been added to this area of research by the recognition that synthetic phosphatases (or more generally hydrolases) have the potential to find application in medicinal and biochemical fields[2] and in the detoxification of chemical weapons and insecticides, as these chemical toxins can also contain phosphate esters.[18] The development of efficient synthetic hydrolases relies on an understanding of the mechanisms by which nature achieves such rate enhancements and of the role played by metal ions and other functional groups present in the active site.[8] The incorporation of vital features of the active sites should then lead to synthetic mimics with improved activity.[7]

Many types of metal complexes have been designed and investigated for their ability to cleave phosphate ester bonds such as those found in BNPP [bis(*p*-nitrophenyl)phosphate],[19,20] RNA,[21−28] DNA,[5,6,19,29−35] and diribonucleotides.[36−40] Metals from the transition series, particularly Zn(II)[19,27,41,42] and Cu(II)[5,6,19,23,24,30,42−45] but also Fe(III)[32] and others,[19,42,46,47]

Table 1. Rate Constants for the Hydrolysis of BNPP by a Selection of Cu(II) Complexes of tacn, Derivatives Thereof, and Some Close Analogues

nucleophile[a]	[complex] (mM)	[BNPP] (mM)	$10^7 k$ (s^{-1})	T (°C)	pH	ref
OH$^-$			4	100	7.2	73
[Cu(tacn)(OH$_2$)$_2$]$^{2+}$	1.3	0.1	3.4	50	7.2	56
[Cu(tacn)(OH$_2$)$_2$]$^{2+}$	1.7	0.015	2.5	50	7.2	71
[Cu(tacn)(OH$_2$)$_2$]$^{2+}$	1.0	1.0	63	50	9.2	8
[Cu([10]aneN$_3$)(OH$_2$)$_2$]$^{2+}$	1.0	1.0	130	50	9.2	8
[Cu([11]aneN$_3$ (OH$_2$)$_2$]$^{2+}$	1.0	1.0	510	50	9.2	8
[Cu$_2$(T$_2$-*o*-X)(OH$_2$)$_4$]$^{4+}$	1.7	0.015	52	50	7.4	71
[Cu$_2$(T$_2$-*m*-X)(OH$_2$)$_4$]$^{4+}$	1.7	0.015	8.6	50	7.4	71
[Cu$_2$(T$_2$-*m*-X Ac$_2$)(OH$_2$)$_2$]$^{2+}$	1.7	0.015	2.1	50	7.4	71
[Cu(*i*Pr$_3$tacn)(OH$_2$)$_2$]$^{2+}$	0.2	1.0	430	50	7.2	54
[Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$	1.7	0.015	630	50	7.4	71
[Cu(Me$_3$tach (OH$_2$)$_2$]$^{2+}$	5.0	1.0	110	50	7.2	74
[(tdciH$_{-3}$)Cu$_2$]$^+$	0.44	1.0	150	25	7.3	75
[Cu(Me$_3$tach)(OH$_2$)$_2$]$^{2+}$	5.0	1.0^b	0.1	50	7.2	74
[Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$	1.7	0.015^b	12	50	7.4	71
[(tdciH$_{-3}$)Cu$_2$]$^+$	1.5	1.0	6	25	7.2	75

^{a} Abbreviations: tacn = [9]aneN$_3$ = 1,4,7-triazacyclononane, [10]aneN$_3$ = 1,4,7-triazacyclodecane, [11]aneN$_3$ = 1,4,8-triazacycloundecane, T$_2$-*o*-X = 1,2-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene, T$_2$-*m*-X = 1,3-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene, T$_2$-*m*-X Ac$_2$ = 1,3-bis(4-(carboxy-methyl)-1,4,7-triazacyclonon-1-ylmethyl)benzene, Me$_3$tacn = 1,4,7-trimethyl-1,4,7-triazacyclononane, *i*Pr$_3$tacn = 1,4,7-triisopropyl-1,4,7-triazacyclononane, Me$_3$tach = *N*,*N*′,*N*-trimethyl-*cis*,*cis*-1,3,5-triaminocyclohexane, tdci = 1,3,5-trideoxy-1,3,5-tris(dimethylamino)-*cis*-inositol, BNPP = bis(4-nitrophenyl)phosphate, NPP = 4-nitrophenyl phosphate. ^{b} NPP concentration.

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as well as from the lanthanide and actinide series [e.g., La(III),[21,24,26,42] Ce(III),[46] Eu(III),[21,24,29,33] Lu(III)[24] and Th(IV)[48]], have been used in combination with a variety of macrocyclic and acyclic polydentate ligands. These studies have identified features of model compounds that contribute to faster phosphate ester cleavage. For mononuclear complexes, two important features to emerge are that the metal center must have a site available for the coordination of the phosphate ester and a water ligand cis to the coordinated phosphate ester, which, upon deprotonation, forms a coordinated hydroxide to act as a nucleophile in attacking the phosphate ester.[49]

Over the past decade or so, recognition that the complexes of 1,4,7-triazacyclonane (tacn), mono- and binucleating derivatives of tacn, and tridentate analogues with larger ring size can fulfill these requirements has led to studies of the ability of such complexes to hydrolyze phosphate esters.[4−8,23,50−55] Table 1 presents a selection of data obtained in recent years for Cu(II) complexes of relevance to the present study, including [Cu(tacn)Cl$_2$], a system studied in

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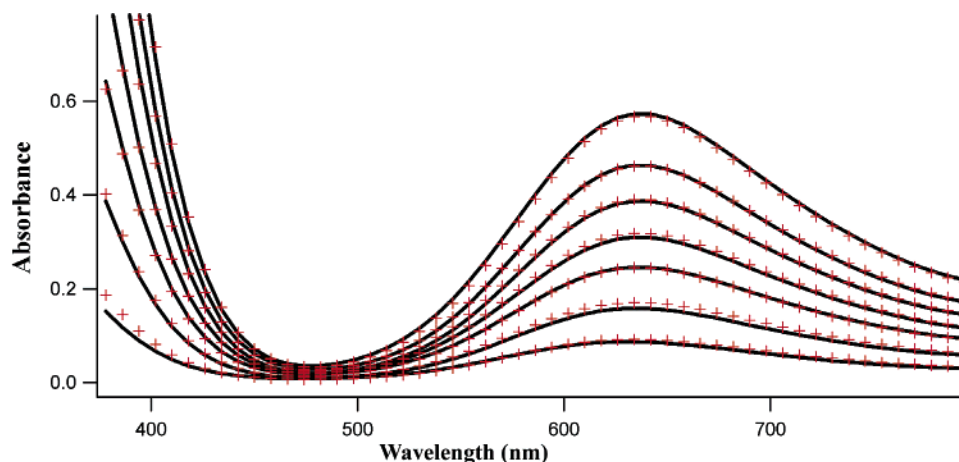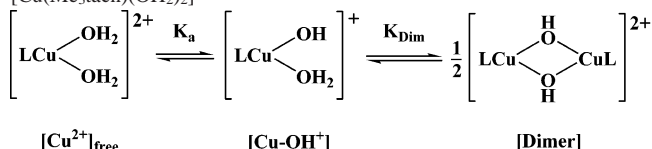
Figure 1. Dependence of the $[Cu(Me_3tacn)(H_2O)_2]^{2+}$ spectrum (lines) on concentration of complex [1.0 (bottom curve), 2.0, 3.0, 4.0, 5.0, 6.0, and 7.5 mM (top curve)] recorded at 65 °C and pH 9.1 with fit to the data shown as +'s.

detail by Burstyn and co-workers.[4−8,56,57] Recently, Burstyn and co-workers reported that the Cu(II) complex of the *N*-alkylated ligand 1,4,7-triisopropyl-1,4,7-triazacyclononane (^{i}Pr$_3$tacn) is a much more effective cleavage agent than the parent Cu(II)−tacn complex.[54] An increase in the rate of cleavage of the model ester BNPP of over 50-fold in the initial rate constants was partly attributable to a reduction in the formation of the "hydrolytically inactive" dihydroxo-bridged complex $[LCu(\mu\text{-}OH)_2CuL]^{2+}$, leading to an increase in the proportion of "active" mononuclear complex.

Our interest in the coordination chemistry of 1,4,7-triazacyclononane derivatives[58−72] led us to examine the

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Scheme 1. Deprotonation and Dimerization of $[Cu(Me_3tacn)(OH_2)_2]^{2+}$

ability of their metal complexes to hydrolyze phosphate esters.[71] Exploratory experiments indicated that the rate of cleavage of BNPP by the Cu(II) complex of 1,4,7-trimethyl-1,4,7-triazacyclononane, $[Cu(Me_3tacn)(OH_2)_2]^{2+}$, was similar to that reported for $[Cu(^iPr_3tacn)(OH_2)_2]^{2+}$ [54] and higher than those for $[Cu(tacn)(OH_2)_2]^{2+}$ [7,56,57] and a variety of complexes of bis(tacn) derivatives.[71] This finding prompted the detailed kinetic study presented herein.

Results

Equilibrium Measurements. The crystallization of the dihydroxo-bridged dimer $[(Me_3tacn)Cu(\mu\text{-}OH)_2Cu(Me_3tacn)]^{2+}$ [76] in solutions used to conduct kinetic runs at higher pH ($>$9) and with higher concentration of complex prompted a determination of the pK_a of $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ and the constant for the dimerization of the conjugate base $[Cu(Me_3tacn)(OH_2)(OH)]^+$ (K_{dim}) by spectrophotometric pH titrations. Visible spectra were recorded at varying concentrations of complex (1.0−7.5 mM), temperatures (42.5−65 °C), and pH values (pH 5.9, 7.0, and 9.1) at a constant ionic strength (I) of 0.15 M (NaClO$_4$). As the concentration of complex and pH was increased, a blue shift and increase in the intensity of the absorbance maxima of the copper(II) d−d transition was observed (see Figure 1 for the dependence on concentration of complex). The spectral data and the finding that the dimer crystallizes at high pH and concentration of complex can be interpreted in terms of the reaction sequence shown in Scheme 1, previously used to account for the behavior of $[Cu(tacn)(OH_2)_2]^{2+}$.[8,56] Details of the data analysis are provided in the Experimental Section. The quality of the fit to the data is highlighted in Figure 1 (+) and the values of K_a and K_{dim} are summarized in Tables 2 and 3.

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Table 2. Acid Dissociation and Dimerization Constants Measured at 50 °C for a Series of Cu(II) Complexes of tacn Derivatives

complex	pK_a	$\log K_{dim}$	ref(s)
$[Cu(Me_3tacn)(OH_2)_2]^{2+}$ [a]	7.85	3.48	this work
$[(Cu\ Me_3tacn)(OH_2)_2]^{2+}$ [b]	7.64	2.30	this work
$[(Cu\ Me_3tacn)(OH_2)_2]^{2+}$ [c]	7.33	3.05	this work
$[Cu(tacn)(OH_2)_2]^{2+}$	7.3	4.1	54, 56
$[Cu(^iPr_3tacn)(OH_2)(OH)]^+$	7.9	3.1	54
$[Cu([11]aneN_3)(OH_2)(OH)]^+$	8.2	3.7	8
$[Cu([10]aneN_3)(OH_2)(OH)]^+$	7.6	3.1	8

[a] From changes in the visible spectrum in the 350−800 nm region. [b] From initial rate kinetic analysis (vide infra). [c] From biphasic kinetic analysis (vide infra).

Kinetic Studies. Experiments were carried out at various temperatures (42.5−65.0 °C), Cu(II)−Me₃tacn complex concentrations (1.0−7.5 mM), and pH values (5.9−9.1 maintained through the use of various buffers). The concentration of BNPP was kept constant (15 μM), such that the complex was in large excess relative to BNPP. The release of p-nitrophenylate (NP) was monitored at 400 nm. A series of absorbance vs time profiles are provided as Supporting Information (Figure S1), which indicate that the rate of cleavage increases with increasing pH and concentration of complex. The profiles, in particular those for pH's 7.4 and 9.1, and total absorbance change indicate a biphasic reaction involving the release of two NP ions initially from BNPP and, once formed, from NPP. Study of the biphasic nature of BNPP cleavage analysis by ^{31}P NMR spectroscopy was undertaken. A solution of composition [BNPP] = 1 mM, [complex] = 4 mM, pH = 7.4, and I = 0.15M (NaClO₄) was prepared and heated to 50 °C. Samples were removed at various times up to 144 h, and after cation-exchange chromatography to remove the paramagnetic Cu(II) complex and concentration of the sample, the ^{31}P NMR spectrum was recorded (see Supporting Information Figure S3). The initial signal of BNPP (at −10.14 ppm relative to phosphoric acid as an external standard) was found to slowly disappear and to no longer be present after 22.5 h. The disappearance of BNPP is accompanied by the appearance of new signal due to NPP in the 2 ppm region. After about 5 h, a signal due to the phosphate appears at 5 ppm and continues to grow very slowly over time.

A check was also carried out on whether the fully protonated complex, $[Cu(Me_3tacn)(OH_2)_2]^{2+}$, is able to hydrolyze BNPP. Under the conditions chosen ([BNPP] = 0.015mM, [complex] = 1 mM, pH = 4.5, I = 0.15 M, T = 50 °C), the complex is fully protonated ($pK_{a1} > 7$ vide supra), and there is no evidence of BNPP cleavage after 5 days.

Kinetic analysis based on the production of two NP ions (see Experimental Section) gave values of two observed rate constants (k_{obs1} and k_{obs2}). Good fits to the data were generally obtained, indicating that, under the reaction conditions used, the release of each NP ion is a first-order process. As found in some previous studies,[2,77] the cleavage of BNPP (k_{obs1}) is on the order of 50−100 times faster than cleavage of NPP (k_{obs2}). k_{obs1} was found to increase with pH, temperature, and

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concentration of complex (Table S1). In general, this also applied to k_{obs2}, but the dependence on the other parameters was less systematic because of instrument instability (baseline drift), particularly for slow reactions that needed to be followed for very long time periods. Because our interest was in the cleavage of the diester BNPP, the kinetic data that we now describe focus primarily on k_{obs1}. Instrumental drift was found to affect the very slow reactions and led us to analyze the data in two ways: (i) fitting to a biphasic equation as indicated above and (ii) following the initial rate method applied by others (for example, see ref 54) and then converting the initial rate data into the corresponding k_{obs1} values. In general, there was good correspondence in the rate constants obtained from the two approaches.

The mass balance equation (eq 1) takes into account each Cu(II) complex that might be present in solution in the absence of the phosphate ester (see Scheme 1)

$$[Cu]_{total} = [Cu-OH_2^{2+}] + [Cu-OH^+] + 2[dimer] \quad (1)$$

where $[Cu]_{total}$, $[Cu-OH_2^{2+}]$, $[Cu-OH^+]$, and [dimer] are the concentrations of the copper complex in total, $[Cu(Me_3tacn)(OH_2)_2]^{2+}$, $[Cu(Me_3tacn)(OH_2)(OH)]^+$, and $[(Me_3tacn)Cu(\mu-OH)_2Cu(Me_3tacn)]^{2+}$, respectively. Application of the equilibria $K_a = [Cu-OH^+][H^+]/[Cu-OH_2^{2+}]$ and $K_{dim} = [dimer]/[Cu-OH^+]^2$, simplification, and rearrangement gives an equation for $[Cu-OH_2^{2+}]$

$$[Cu-OH_2^{2+}] = \frac{-\left(1 + \dfrac{K_a}{[H^+]}\right) + \sqrt{\left(1 + \dfrac{K_a}{[H^+]}\right)^2 + \dfrac{8K_{dim}K_a^2[Cu]_{total}}{[H^+]^2}}}{\dfrac{4K_{dim}K_a^2}{[H^+]^2}} \quad (2)$$

Of the three complexes that might be present in solution, $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ and $[Cu(Me_3tacn)(OH_2)(OH)]^+$ may bind and hydrolyze BNPP (Scheme 2), and as concluded for the other Cu-tacn systems,[7,8,56] the dimer is expected to be inactive because coordination of BNPP would require cleavage of a hydroxide bridge and, in addition, bridging hydroxides are poorer nucleophiles than terminal hydroxides.

The rate law for cleavage of BNPP is given by

$$-\frac{d[BNPP]}{dt} = k_1[Cu-OH_2(BNPP)^+] + k_2[Cu-OH(BNPP)] \quad (3)$$

Substituting the appropriate expressions for k_1 and k_2 yields

$$-\frac{d[BNPP]}{dt} = (k_1K_1[Cu-OH_2^{2+}] + k_2K_2[Cu-OH^+])[BNPP] \quad (4)$$

Because the reaction is followed under pseudo-first-order

Table 3. Rate Constants and Thermodynamic Parameters Derived from Fitting the Observed Rate Constant Data (k_{obs1}) to Eq 7 and Spectrophotometric pH Titration Data

T (°C)	$k_2K_2{}^a$ (M^{-1} s^{-1})	$k_2K_2{}^b$ (M^{-1} s^{-1})	p$K_a{}^a$	p$K_a{}^b$	p$K_a{}^c$	log $K_{dim}{}^a$	log $K_{dim}{}^b$	log $K_{dim}{}^c$
42.5	0.035	0.062	7.86	7.88	7.68	2.09	2.53	2.82
50.0	0.060	0.125	7.64	7.33	7.85	2.30	3.05	3.48
57.5	0.114	0.196	7.48	7.43	7.23	2.62	2.70	2.04
65.0	0.148	0.272	7.64	7.55	7.01	2.00	2.24	1.50

^{a} k_{obs1} from initial rate data. ^{b} k_{obs1} from fit of absorbance data to biphasic kinetic equation. ^{c} From spectrophotometric pH titration data.

Scheme 2
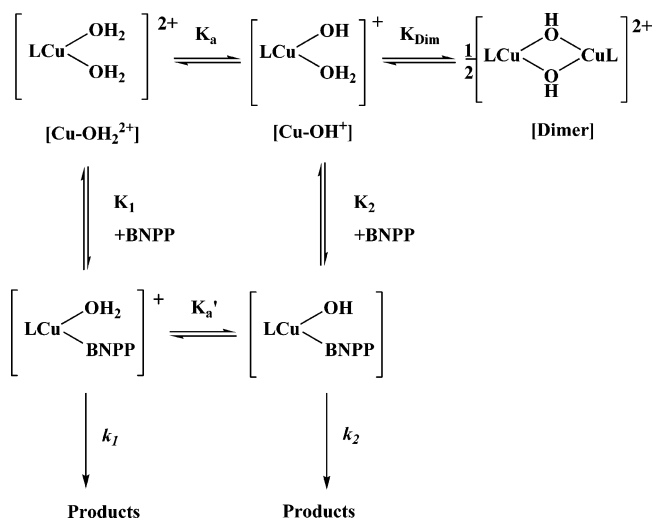
conditions with excess Cu(II) complex, the following expression for k_{obs1} can be derived

$$k_{obs1} = \left(k_1K_1 + k_2K_2\frac{K_a}{[H^+]}\right)[Cu-OH_2{}^{2+}] \quad (5)$$

Substitution of eq 2 into eq 5 then gives the following expression

$$k_{obs1} = \left(k_1K_1\frac{[H^+]}{K_a} + k_2K_2\right)\left\{\frac{1}{4K_{dim}}\left[-\left(\frac{[H^+]}{K_a}+1\right)+\sqrt{\left(\frac{[H^+]}{K_a}+1\right)^2 + 8K_{dim}[Cu^{2+}]_{total}}\right]\right\} \quad (6)$$

The initial intention was to use the experimentally determined values of K_a and K_{dim} to aid in the elucidation of the hydrolytically active species and the second-order rate constants; however, it was found that better fits to the data were obtained when these parameters were determined as part of the analysis. Fitting of the data to eq 6, using the MATLAB[78] programming environment, gave values of k_1K_1, k_2K_2, K_a, and K_{dim}. It was found that the uncertainty in k_1K_1 was greater than the fitted value. An experiment carried out at pH 4.5 confirmed that the rate of cleavage of BNPP by the diaqua complex is too low to be measured (vide supra) under the conditions of our study and does not contribute to the reaction rate. The k_{obs1} data obtained from initial rate and biphasic kinetic analysis was therefore fitted to eq 7;

values of k_2K_2, K_a, and K_{dim} were determined (Table 3); and activation parameters were calculated (Table 4). Residual plots indicated that K_{dim}, in particular, has large uncertainties. Typical fits of the k_{obs1} data obtained from initial rate analysis to eq 7 are shown in the Supporting Information (Figure S2).

$$k_{obs1} = k_2K_2\left\{\frac{1}{4K_{dim}}\left[-\left(\frac{[H^+]}{K_a}+1\right)+\sqrt{\left(\frac{[H^+]}{K_a}+1\right)^2 + 8K_{dim}[Cu^{2+}]_{total}}\right]\right\} \quad (7)$$

Binding of BNPP. The constants for the binding of BNPP to [Cu(Me₃tacn)(OH₂)₂]²⁺ and [Cu(Me₃tacn)(OH₂)(OH)]⁺ (K_1 and K_2) are the product of an ion pairing constant (K_{IP}) and binding constant (K_{bind}), i.e., $K_{1\ or\ 2} = K_{IP}K_{bind}$. The Fuoss−Davies equation[81,82] predicts K_{IP} values of ~1 and 40 M^{-1} between +1 and −1 and +2 and −1 species, respectively,[83] and under the conditions of our study, less than 1% of BNPP would be ion-paired. An attempt to determine K_1 and K_2 by measuring the rate of BNPP cleavage at a constant [Cu(Me₃-tacn)(OH₂)₂]²⁺ concentration of 0.3 mM and increasing concentration of BNPP (up to 10 mM, limited by BNPP solubility) found that the dependence of k_{obs} on concentration of BNPP is linear. Because the plot did not show saturation behavior (i.e., was not curved within the accessible range of BNPP concentrations), the Michaelis constant[56] could not be determined from the data.

Catalytic Turnover. The complex [Cu(Me₃tacn)(OH₂)₂]-(ClO₄)₂ was tested for the ability to act as a catalyst, and not merely as a "rate enhancer", by conducting a series of experiments at pH 5.9, 7.4, and 9.1 and 50 °C with excess BNPP (1 mM) and variable concentration of complex and BNPP/Cu ratio. The release of NP was followed for several weeks, again by monitoring of the spectral change at 400 nm (Figure 2). The molar extinction coefficient of NP determined at each pH [pK_a(HNP) = 7.15[72]] was used to determine the concentrations of NP from which turnover numbers were calculated (Table 5).

Discussion

Our recent finding that the Cu(II)−Me₃tacn complex cleaves BNPP at a higher rate than the Cu(II) complexes of

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Table 4. Rate Constants and Activation Parameters for the Hydrolysis of BNPP by $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ and by a Selection of Mononuclear Metal Complexes

nucleophile	$10^4 k$ (50 °C)	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta G^{\ddagger i}$ (kJ mol^{-1})	ref
$[Cu(Me_3tacn)(OH_2)(OH)]^+$	650[a]	56 ± 6	−95 ± 18	87 ± 10	this work
$[Cu(Me_3tacn)(OH_2)(OH)]^+$	1350[b]	55 ± 6	−92 ± 20	85 ± 10	this work
$[Cu(Me_3tacn)(OH_2)_2]^{2+}$					this work
(pH = 7.4)[c]	0.36	69 ± 8	−118 ± 26	107 ± 16	
(pH = 7.4)[d]	0.66	76 ± 3	−89 ± 8	105 ± 6	
$[Cu(Me_3tacn)(OH_2)_2]^{2+}$					this work
(pH = 9.1)[c]	0.83	58 ± 2	−144 ± 6	105 ± 4	
(pH = 9.1)[d]	1.1	62 ± 1	−129 ± 2	104 ± 2	
$[Cu(^iPr_3tacn)(OH_2)(OH)]^+$	600[e]	51 ± 2	−110 ± 6	87 ± 4	54
$[Cu(tacn)(OH_2)(OH)]^+$	2[e]	85	−79	111	57
$[Ir(en)_2(OH_2)(ENPP)]^{+ f}$	6.5	69	−93	99	49
$[Co(en)_2(NPP)(OH)]^{+ g}$	91	71	−65	92	79
OH$^-$	0.000003[h]	104	−106	138	80

[a] Second-order rate constant from initial rate measurements in units of M^{-1} s^{-1}. [b] Second-order rate constant from biphasic kinetic analysis in units of M^{-1} s^{-1}. [c] Calculated from observed rate constants determined from initial rate analysis at pH indicated and [complex] = 2 mM in units of s^{-1}. [d] Calculated from observed rate constants determined from initial rate analysis at pH indicated and [complex] = 2 mM in units of s^{-1}. [e] 1.5-order rate constant, $k_{1.5}$, in units of M$^{-1/2}$ s^{-1}. [f] Parameters are for the hydrolysis of coordinated ENPP (ethyl-4-nitrophenyl phosphate). [g] Parameters are for the hydrolysis of coordinated NPP. [h] First-order rate constant measured in units of s^{-1}. [i] Calculated at 50 °C.
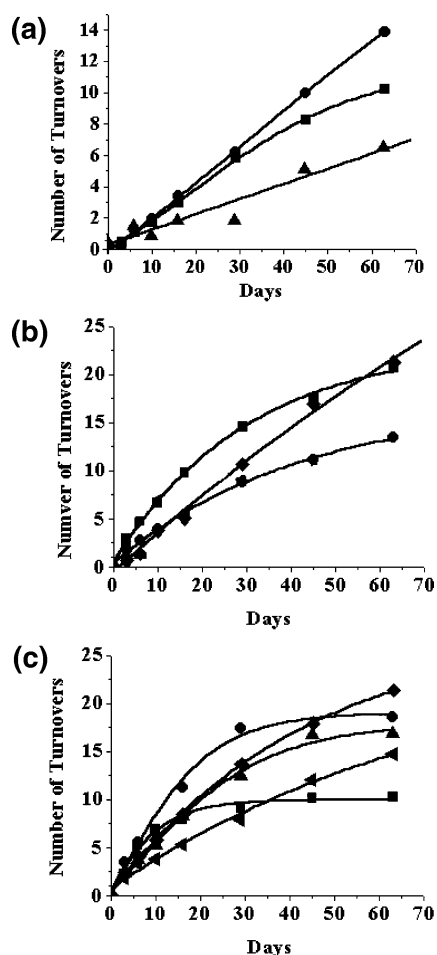

Figure 2. Plots of turnover numbers vs time for catalytic experiments carried out at various $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ concentrations and pH values and at [BNPP] = 1.0 mM and T = 50 °C. pH: (a) 5.9, (b) 7.4, and (c) 9.1. [complex]: ■ 100, ▲ 25, ● 10, ♦ 2.5, ▼ 1 μM. Solid lines highlight the trend.

Table 5. Turnover Numbers in the First 6 Days and after 63 Days (in Parentheses) of Experiments Conducted at 50 °C at Various Concentrations of Complex and pH's, [BNPP] = 1 mM, and I = 0.15 M

[Cu]/[BNPP] ratio	pH 5.9	pH 7.4	pH 9.1
0.1	1.1 (10)	5.2 (13)	5.3 (10)
0.025	1.2 (14)	4.8 (21)	5.9 (19)
0.01	1.0 (6.0)	2.8 (13)	3.8 (17)
0.001	nd[a]	nd[a]	3.4 (15)

[a] Absorbance changes were too small to enable determination of turnover numbers.

of cleavage of NPP to inorganic phosphate. The kinetic analysis was facilitated by the fact that cleavage of BNPP is ca. 50−100 times faster than cleavage of NPP. Deal and Burstyn[56] found a similar variation in their study of the [Cu(tacn)(OH$_2$)$_2$]$^{2+}$ complex. Comparisons of the first-order rate constants (k_{obs}) determined in this study to those reported previously are limited by the variety of conditions and types of phosphate esters that have been used. Apart from BNPP, other commonly used diesters include 2-hydroxypropyl-4-nitrophenyl phosphate (HPNP),[84] 2,4-dinitrophenylethyl phosphate (DNPEP),[75] and ethyl-4-nitrophenyl phosphate (ENPP).[56,74] Two comparisons of the reactivity of BNPP and ENPP, reported by Planalp and co-workers[74] for [Cu(Me$_3$-tach)(OH$_2$)$_2$]$^{2+}$ (Me$_3$tach N,N',N''-trimethyl-cis,cis-1,3,5-triaminocyclohexane) and Deal and Burstyn[56] for [Cu(tacn)-(OH$_2$)$_2$]$^{2+}$, indicate that the cleavage of BNPP is ca. 15-fold faster than cleavage of ENPP. Consequently, the faster-reacting BNPP was chosen in preference to ENPP.

The selection of observed rate constants for BNPP hydrolysis, presented in Tables 1 and 4, confirm that the rate of cleavage of BNPP by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ is higher than those for most other complexes of divalent metal ions. Comparison with the related system [Cu(tacn)(OH$_2$)$_2$]$^{2+}$ shows that N-methylation of the tacn ring increases the rate significantly. For [Cu(tacn)(OH$_2$)$_2$]$^{2+}$ at pH 7.24, 1.3 mM complex, and 0.1 mM substrate,[56] the rate of cleavage is ca.

various binucleating tacn derivatives[71] prompted a more detailed kinetic investigation of this reaction. In contrast to many previous studies, the reaction could be followed for sufficient time to allow the determination of rate constants for the cleavage of BNPP to NPP and estimates of the rate

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Table 6. Selection of First-Order Rate Constants for the Hydrolysis of NPP

complex[a]	[complex] (μM)	[substrate] (μM)	pH	T (°C)	$10^6 k$ (s^{-1})	ref
[Cu(Me$_3$tacn)(H$_2$O)$_2$]$^{2+}$	3000	15	7.4	50	1.93	this work
[Cu(Me$_3$tacn)(H$_2$O)$_2$]$^{2+}$	3000	45	7.4	50	2.28	this work
[(tdciH$_{-3}$)Cu$_2$]$^+$	3000	3000	8.4	25	1.98	75
cis-[Co(en)$_2$(OH$_2$)NPP]	75	500	9−11.8	25	780	79
[Zn$_2$L^1]$^{3+}$	5000	10,000	5.9	35	7.5	85
[Zn$_2$L^2]$^{4+}$	230	230	7.6	55	2.8	40
[Cu(tren)]$^+$	100	500	8.0	75	5.2	86
[Ni(tren)]$^{2+}$	100	500	8.0	75	5.6	86
H$_2$O	−	−	−	25	0.0002	87

[a] Abbreviations: L^{1}H = (26-hydroxy-1,4,7,10,13,16,19,22-octaazabicyclo[11.11.3]heptacosane, L^2 = S, S′-bis(10-(1,4,7-triazacyclodidecyl))-1′,3′-dithiobenzene, tren = tris(2-aminoethyl)amine, tdci = 1,3,5-trideoxy-1,3,5-tris(dimethylamino)-*cis*-inositol.

200 times lower than that of cleavage by [Cu(Me$_3$tacn)-(OH$_2$)$_2$]$^{2+}$ at the same temperature and slightly different reaction conditions (pH 7.4, 1.7 mM complex and 15 μM BNPP). A slightly smaller enhancement has been observed for [Cu(^{i}Pr$_3$tacn)(OH$_2$)$_2$]$^{2+}$ relative to the same tacn complex. Cleavage of BNPP by [Cu(Me$_3$tach)Cl$_2$] complex is ca. 50 times faster than cleavage by [Cu(tacn)(OH$_2$)$_2$]$^{2+}$. For the Me$_3$tach complex, meaningful comparison with the behavior of the complex of the nonalkylated ligand (*cis,cis*-1,3,5-triaminocyclohexane) was precluded by the lack of data on the latter. In general, the available data do indicate that alkylation enhanced cleavage rates.

When compared with small-molecule phosphatase mimics based on other divalent metal ions, such as Zn(II) and Ni(II), Cu(II) complexes are generally more effective. A number of complexes of trivalent metal ions, such as Co(III) and Eu(III), cleave phosphate esters at significantly higher rates than the divalent metal complexes. These metal ions are harder Lewis acids than Cu(II), because of the increased charge-to-radius ratio, and therefore, they exhibit greater electrostatic interactions with the phosphate esters.[56] It has been previously pointed out, however, that kinetically inert Co(III) complexes do not function as catalysts because of low rates of product release or substrate attachment.[57]

Comparisons of k_{obs2} with literature data are mainly confined to studies that have used NPP directly, as there are few reports of the biphasic hydrolysis of BNPP, and are further limited by the different temperatures used (Table 6). Nevertheless, it is clear that the rate of hydrolysis of NPP by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ is similar to that achieved with other divalent metal complexes and, in contrast to the rates of BNPP cleavage, NPP hydrolysis rates show relatively little variation (only 1−2 orders of magnitude across the series of complexes).

The contrast in the dependence of the BNPP and NPP cleavage rates on the complex used is notable and indicates that cleavage agents that are very effective in hydrolyzing phosphate diesters yet ineffective toward phosphate monoesters can be developed. The reasons for the difference in reactivity are unclear, but we have previously shown that

phosphate monoesters and related organic phosphates can form stable bridged complexes on reaction with mononuclear and binuclear complexes of tacn derivatives, which can be crystallized from solution. For example, the trinuclear complex [Cu$_3$(Me$_3$tacn)$_3$(Ph−PO$_3$)$_2$](ClO$_4$)$_2$·^{1}/$_2$H$_2$O was obtained from the reaction of [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ with phenyl phosphate (Ph−PO$_3$$^{2-}$), a close analogue of NPP, and the binuclear complex [Cu$_2$(T$_2$-*m*-X)(NPP)(μ-OH)](ClO$_4$)·H$_2$O was obtained from the reaction of NPP with [Cu$_2$(T$_2$-*m*-X)(OH$_2$)$_4$]$^{4+}$, where T$_2$-*m*-X = 1,3-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene.[72] Even though the first product does not incorporate a phosphate monoester, the two structures highlight the possibility that "fully saturated" complexes can be formed that cannot generate a terminal hydroxyl group to attack the phosphate ester and essentially protect the ester from intramolecular attack. The higher charge of NPP (2−; cf. 1− for BNPP) and higher number of oxygens that can potentially bind to metal centers clearly increases the affinity of this anion for a metal ion (see ref 11 and references therein). Coordination of NPP or Ph−PO$_3$$^{2-}$ could involve either chelation or the formation of bridged complexes (preferred in the cited examples), both of which can lead to hydrolytic inactivation. For the binuclear complex, the bridging OH$^-$ can potentially attack the phosphate ester but is less nucleophilic than a terminal hydroxide. Hydrolysis of the coordinated ester could still occur via the less effective intermolecular attack by OH$^-$. Moreover, the presence of two negative charges on NPP makes the P center less electropositive and less prone to nucleophilic attack.

The cleavage of BNPP by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ is proposed to involve the equilibria and kinetic processes outlined in Scheme 2, with the catalytically active agent being [Cu(Me$_3$tacn)(OH$_2$)(OH)]$^+$, in keeping with the findings of previous investigations of Cu(II)−tacn derivatives.[54,56,57] Assuming that common pathways are involved in the cleavage of BNPP by these complexes, the higher rate of cleavage promoted by [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$, cf. [Cu(tacn)-(OH$_2$)$_2$]$^{2+}$, could arise from (i) an increase in the concentration of the conjugate base arising from either an increased acidity of the complex (pK_a) or a decreased constant for dimerization (K_{dim}), (ii) an increase in the constant for binding of BNPP to the conjugate base, and/or (iii) an increase in the rate of cleavage of the Cu(II)-bound phosphate diester. The cleavage reaction can be treated as a two-step process involving (i) coordination of BNPP to Cu(II), followed by

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(87) Kirby, A. J.; Jenks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 3209.

(ii) intramolecular cleavage, rather than an intermolecular process, in which a BNPP···Cu(II) complex ion pair is formed and facilitates attack of the ester by coordinated OH$^-$. Unfortunately, in this study, the BNPP binding constant could not be measured under the accessible reaction conditions (limited by the solubilities of the components), but experimental values of pK_a and K_{dim} were able to be determined both directly (pH dependence of UV−visible spectrum) and indirectly (kinetics).

The pK_a and K_{dim} data for [Cu(Me$_3$tacn)(OH$_2$)$_2$]$^{2+}$ and related complexes (Table 2) show variations of less than 1 order of magnitude, and in each, the higher acidity is generally coupled with higher K_{dim}. This means that the two parameters are compensatory in that the more acidic complexes also seem to have an increased ability to form the hydrolytically inactive dimer. Therefore, at any chosen pH and complex concentration, the proportion of hydrolytically active conjugate base does not vary sufficiently across the series of Cu(II)−tacn complexes studied to account for the observed rate changes. Thus, it can be concluded that the observed variations in rate arise from changes in the affinity of BNPP for the Cu(II) complex (K_1 or K_2 in Scheme 2) or in the rate of BNPP cleavage following coordination (k_1 or k_2 in Scheme 2). The further analysis of the observed rate constant, k_{obs1}, established that the pathway involving coordination attack of BNPP (K_1k_1) by coordinated water does not make a measurable contribution to the reaction rate (i.e., cleavage within [Cu(Me$_3$tacn)(BNPP)OH$_2$]$^+$ is comparatively slow). For the remaining pathway (K_2k_2), the process for the binding of BNPP to Cu(II) could not be separated from actual attack on and cleavage of BNPP. As a consequence, composite values of K_2k_2 were determined. Because each Cu−tacn complex has the same charge, the charges of the interacting species [Cu(II) complex and BNPP] are always the same. Therefore, in the absence of, for example, noncoordinative interactions (van de Waals, dispersion forces, etc.), the bulkier alkyl groups might be expected to lead to a decrease in binding affinity, especially so for isopropyl groups. This would mean that K_2 will be constant, or more likely decrease, with increasing bulk of the tacn substituents and that the reaction should be slower. It can therefore be argued that, because k_{obs1} is directly proportional to K_2k_2, the substantial increase in reactivity that accompanies alkylation (viz., highest rates of BNPP cleavage are exhibited by [Cu(Me$_3$tacn)(OH$_2$)(OH)]$^+$ and [Cu(^{i}Pr$_3$tacn)(OH$_2$)-(OH)]$^+$; see Tables 1 and 4) is associated with intramolecular nucleophilic attack of BNPP by a coordinated hydroxide ion. The rate enhancements can be considered to be a reflection of changes in k_2. The increases in k_2 are sufficient to offset any decrease in the affinity of the ester for the Cu(II) complex (i.e., smaller K_2 value) that might accompany the introduction of bulkier substituents.

Although [Cu(Me$_3$tacn)(OH$_2$)(OH)]$^+$ and [Cu(^{i}Pr$_3$tacn)-(OH$_2$)(OH)]$^+$ exhibit higher rates of BNPP cleavage than any other Cu(II)−tacn systems studied so far (Table 4), there are recent reports of Cu(II) complexes that are more effective cleavage agents. For example, Gajda et al.[75] reported that the binuclear "[(tdciH$_{-3}$)Cu$_2$]$^+$" complex exhibits a much higher rate of BNPP cleavage, which is conveniently measured at room temperature. The Me$_3$tach complex, on the other hand, is slightly less effective.[74]

Few studies have reported the determination of activation parameters for the observed and higher-order rate constants. A selection of data is presented in Table 4. A comparison of the $\Delta H^\ddagger$ values for the three tacn systems (tacn, ^{i}Pr$_3$tacn, and Me$_3$tacn) reveals that the observed rate increases are accompanied by significant decreases in the values of $\Delta H^\ddagger$. The contribution of $\Delta H^\ddagger$ to the activation free energy, $\Delta G^\ddagger$, decreases in going from the tacn complex (77%) to the other complexes (59−65%). In terms of reaction mechanism, it has previously been established, through careful isotopic labeling experiments,[57] that although the values of $\Delta S^\ddagger$ are negative, the cleavage of BNPP by the Cu(II)−tacn complex follows a concerted mechanism rather than a fully associative mechanism, i.e., the reaction proceeds via a five-coordinate transition state in which bond formation with the attacking coordinated OH$^-$ is accompanied by weakening of the P−OPhNO$_2$ bond. This conclusion is reasonable given that, in all cases, the attachment of BNPP to Cu(II) will result in a negative contribution to $\Delta S^\ddagger$ (cf. relative size of the BNPP being coordinated and the water being released) that should dominate over $\Delta S^\ddagger$ components associated with the formation of the transition state. In this context, previous determinations of the activation parameters for intramolecular hydrolysis of NPP and ENPP by OH$^-$ coordinated to Co(III) and Ir-(III), respectively, are instructive.[49,79] For both *cis*-[Co(en)$_2$-(OH)(O$_3$POPhNO$_2$)] and *cis*-[Ir(en)$_2$(OH)(O$_2$P(OEt)(OPh-NO$_2$))]$^+$, $\Delta S^\ddagger$ is negative [−65(5) and −93(5) J K^{-1} mol^{-1}, respectively] and is indicative of a concerted mechanism with significant associative character [more so for the Ir(III) complex]. There is close correspondence between these values and those for three Cu(II)−tacn complexes, viz., [Cu-(tacn)(OH$_2$)(OH)]$^+$, [Cu(^{i}Pr$_3$tacn)(OH$_2$)(OH)]$^+$. and [Cu(Me$_3$-tacn)(OH$_2$)(OH)]$^+$ (see Table 4). It should be noted that the 1.5-order rate constant used to determine activation parameters for the tacn and ^{i}Pr$_3$tacn complexes was calculated slightly differently than the second-order rate constant for Me$_3$tacn. Even so, the $\Delta S^\ddagger$ data support the view that the rate enhancements are largely a reflection of the attack of the coordinated ester by the coordinated OH$^-$ group and that the reaction mechanism increases in associative character in the order tacn < Me$_3$tacn < ^{i}Pr$_3$tacn. It can be argued that the transition state is more compact than the initial state [both OH and BNPP coordinated to Cu(II)] and is stabilized relative to the initial state when alkyl substituents are introduced. However, the activation parameters need to be interpreted with caution as they relate to a composite rate constant that includes contributions from the binding of the ester to the complex (K_2). Thus, it is not absolutely clear whether the changes in entropy of activation reflect changes in reaction mechanism or K_2 for the binding of BNPP to Cu(II). Cone angle calculations for a series of triazamacrocycles based on published structures found, not unexpectedly, that ^{i}Pr$_3$tacn has the greatest steric bulk.[54] A notable finding that has some relevance here is that a number of Cu(II) complexes of ^{i}Pr$_3$tacn and Me$_3$tacn have been isolated in

which small anions such as nitrite and nitrate have formed four-membered chelate rings;[88,89] presumably this has been encouraged by the alkyl substituents.

Catalytic Turnover. The data in Table 5 clearly show that $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ is able to catalyze the hydrolysis of BNPP under all conditions studied. The curved dependence of the total turnover versus time plots for the higher complex concentrations, however, indicates that the rate of cleavage of BNPP decreases with time, especially at the higher concentrations of the complex (see Figure 2). This originates from a reduction in the concentration of either (i) the substrate relative to the complex concentration, viz., only 10 turnovers are possible at the highest concentration of complex (more turnovers are observed at pH's 5.9 and 7.4 because of a contribution from the cleavage of NPP), or (ii) the hydrolytically active complex caused by an increase in the concentration of the dinegatively charged NPP ion as the reaction proceeds, which will bind to the Cu(II) complex more strongly than BNPP. Over the first 6 days of the experiment, a maximum turnover rate of ~1 turnover per day was achieved, and the maximum number of turnovers achieved over the 63-day period was 21. As a general trend, over the first 6 days, the number of turnovers was found to increase with the concentration of complex and pH. However, over this period, there is little difference in activity for the higher concentrations of complex (0.025 and 0.1 mM), and as expected, these concentrations afford the highest turnover rates. Catalytic turnover of BNPP by $[Cu(tacn)Cl_2]$ has been confirmed by Burstyn and co-workers[77] at pH 7.24 (HEPES) and a 5 mM concentration of BNPP, but the actual rate of turnover was not reported. Hay et al.[3] reported a much higher turnover rate and at lower temperature (2.2 turnovers h^{-1} at pH 7.0 and 25 °C) for the reaction of $[Cu(tacn)Cl_2]$ with the 2,4-dinitrophenyldiethyl phosphate triester. An impressive catalytic turnover rate of 32 per day at pH 7 and 25 °C was reported recently by Gajda et al.[75] for $[(tdciH_{-3})Cu_2]^+$, a complex that, unlike the Cu(II)−tacn systems, utilizes two Cu(II) centers to cleave the esters. This finding is stimulating the further development of hydrolytically active polynuclear complexes, including those formed by derivatives of tri-azamacrocyclic ligands.[50−53,71]

Conclusion. The $[Cu(Me_3tacn)(OH)(OH_2)]^+$ complex has been shown to effectively enhance phosphodiester hydrolysis, with the second-order rate constant calculated to be 0.15 M^{-1} s^{-1} at 50 °C, and to exhibit catalytic turnover, with a maximum of 1 turnover per day. The rate of BNPP cleavage is significantly higher than for other mononuclear Cu(II) complexes reported in the literature to date, with the exception of the close analogue $[Cu(^iPr_3tacn)(OH)(OH_2)]^+$, which has been found to exhibit similar cleavage rates. Complexes that exhibit larger rate constants are generally polynuclear or contain harder Lewis acids, such as Co(III) and Ir(III).[7,15,49,55,79] A comparison of the system studied in

this work to enzymes such as alkaline phosphatase shows that, although this complex is one of the most efficient studied for a divalent metal complex, its rate enhancement of the hydrolysis of phosphodiesters is several orders of magnitude lower than that exhibited by natural systems. Nevertheless, the promising rate enhancements observed for the Cu(II) Me_3tacn and $^iPr_3tacn$ complexes have stimulated ongoing investigations of the hydrolytic properties of complexes formed by the tridentate macrocyclic ligand family.

Experimental Section

Materials and Reagents. 1,4,7-Trimethyl-1,4,7-triazacyclononane (Me_3tacn), kindly donated by Unilever, was distilled prior to use. HEPES [N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid], CHES [2-(N-cyclohexylamino)ethanesulfonic acid], MES [2-(N-morpholino)ethanesulfonic acid], MOPS [3-(N-morpholino)propanesulfonic acid], and TAPS [N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] were purchased from Sigma. Other chemicals were of AR grade, and water was distilled prior to use. Sodium perchlorate solutions were prepared by neutralizing standardized sodium hydroxide solution with perchloric acid.

Caution: Although no problems were encountered in this work, metal perchlorate complexes and perchloric acid are potentially explosive. They should be handled with care, and the complexes should be prepared in small quantities.

Synthesis of $[Cu(Me_3tacn)(OH_2)_2](ClO_4)_2$. To an aqueous solution of copper perchlorate hexahydrate (11.51 g, 31 mmol) was added Me_3tacn (5.31 g, 31 mmol) dissolved in 50 mL of a 1:1 ethanol/water mixture. The resulting dark blue solution was adjusted to pH 6 with 2 M NaOH and taken to dryness. The residue was then dissolved in EtOH, and Et_2O was added until the solution became cloudy. The flask was left at −20 °C overnight, after which blue crystals had formed. The product was collected by filtration in vacuo, washed with Et_2O, and air-dried. Yield = 11.48 g (79%). Anal. Calcd for $CuC_9H_{25}N_3O_{10}Cl_2$: C 23.0, H 5.3, N 8.9%. Found: C 23.1, H 5.4, N 8.9%. UV−visible−NIR spectrum [H_2O; λ_{max}, nm (ϵ, M^{-1} cm^{-1})]: 270 (3760), 682 (36), 1237 (20). IR spectrum (KBr; ν, cm^{-1}): 3424 br m, 2982 w, 2908 m, 2856 m, 2812 w, 1638 w, 1498 w, 1451 m, 1298 m, 1145 s, 1090 s, 1011 m, 940 w, 894 w, 781 w, 745 w, 628 m.

Instruments. All UV−visible spectra and kinetic runs were measured using 1-cm cuvettes (quartz or glass) on a Varian Cary 5G spectrophotometer equipped with a jacketed cell holder whose temperature was regulated (±0.1 °C) by an external circulating water bath (Varian). pH was measured on a Metrohm 605 pH meter fitted with an Orion electrode. IR spectra were measured using KBr disks on a Perkin-Elmer 1640 FTIR spectrometer at a resolution of 4.0 cm^{-1}. ^{31}P NMR spectra were recorded in D_2O on a Bruker DPX300 NMR spectrometer with an external reference of phosphoric acid.

Solution Preparation. A series of buffer solutions, prepared by methods described at http://www.bi.umist.ac.uk/users/mjfrbn/Buffers/Makebuf.asp, were used to prepare solutions of constant ionic strength (0.15 M) for kinetic runs by diluting with equal volumes of $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ and BNPP stock solutions. In general, a solution of the free acid was adjusted to the desired pH value by titration with 1 M NaOH and made up to a known volume in a volumetric flask [total concentration of buffer species, 0.150 M; I_{TOT}, 0.382 M (NaClO_4)]. The following buffers were used: MES (pH 6.3), MOPS (pH 7.0), HEPES (pH 7.4), TAPS (pH 8.2), and CHES (pH 9.1).

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Solutions of $[Cu(Me_3tacn)(H_2O)_2]^{2+}$ (3.0−22.5 mM) were made to 3 times the final concentration used in kinetic studies, and a standardized $NaClO_4$ solution (1.044 M) was used to adjust the ionic strength of each solution to 67.5 mM.

A 45 μM aqueous stock solution of BNPP was prepared from NaBNPP. Solutions of BNPP used in saturation experiments were diluted from a 12.0 mM stock solution in cases where the final concentration of BNPP was ≤4.0 mM, whereas for higher concentrations, solid NaBNPP was added directly to the complex/ buffer solution. A 3.0 mM BNPP solution was used in catalytic turnover experiments that, when diluted with buffer and complex solutions, gave a BNPP concentration of 1.0 mM.

Spectrophotometric Data Measurement and Analysis. (i) **Equilibrium Studies.** The analysis of the spectrophotometric data involved a two-step procedure. A model-free principle component analysis (PCA) was used to establish the number of factors needed to describe the spectra to within the experimental uncertainty,[90] and as would be expected for the reaction sequence shown in Scheme 1, it was found that three components were required. The spectra were then modeled using nonlinear least-squares regression using two optimization algorithms as described in detail by Brügger et al.[90] to determine K_a and K_{dim} and the molar extinction coefficient for each species. The latter was optimized using a quadratic programming method, while the values of K_a and K_{dim} were varied using a simplex algorithm[91] to minimize a residual function based on the sum of the squares of the difference between measured and calculated absorbances.[91] An example of the quality of the fit to the experimental data is shown in Figure 1 (+). The calculated values of K_a and K_{dim} are reported in Tables 2 and 3.

(ii) Kinetic Studies. In a typical experiment, an aliquot of a stock solution of the copper complex (3.0−22.5 mM) at $I = 67.5$ mM ($NaClO_4$) was added to an equal volume of a buffer solution in a 1 cm cuvette, and the temperature was allowed to equilibrate in the spectrophotometer cell block for 10 min. An equal volume of the BNPP stock solution (45 μM) was then added to the cuvette, the solution mixed thoroughly, and the temperature was allowed to equilibrate for a further 2−3 min before readings commenced. The final concentrations were as follows: [BNPP], 15 μM; [complex], 1.0−7.5 mM; [buffer], 50 mM; pH range, 6.3−9.1; and I, 0.15 M. Kinetic runs were carried out at four different temperatures in most cases, i.e., 42.5, 50.0, 57.5 and 65.0 °C (±0.1 °C). Reactions were followed by monitoring the production of p-nitrophenylate (NP) at 400 nm for up to 8000 min. Absorbance readings were taken every 5 min. An experiment was also carried out at pH 4.5 and 50 °C to check on the ability of the protonated complex $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ to cleave BNPP. A solution with composition [BNPP] = 0.015 mM, [complex] = 1 mM, pH = 4.5, and $I = 0.15$ M was prepared, and the production of NP was again monitored spectrophotometrically for over 5 days.

An additional experiment was conducted to elucidate the biphasic nature of NP release. A solution of BNPP (3 mM, 100 mL) was mixed with a MOPS buffer solution [pH 7.4 at 50 °C, $I = 0.383$ M ($NaClO_4$)]. The resulting solution was heated to 50 °C and maintained at this temperature for 20 min prior to the addition of the copper(II) complex solution (12 mM, 100 mL). Timing commenced once the Cu(II) had been added. A 50 mL aliquot of the reaction mixture was taken after 1.5, 5.5, 22.5, 49, and 144 h; cooled to room temperature; and loaded onto a Sephadex SP C25 cation-exchange column (Na^+ form). The copper(II) complex was immobilized on the column, and a single yellow band passed through the column and was fully removed upon washing with water. The yellow fraction was taken to dryness under vacuum at low temperature (50 °C)—both BNPP and NPP hydrolyze extremely slowly under these conditions—dissolved in D_2O, and analyzed by ^{31}P NMR spectroscopy. The experiment confirmed that the complex is able to cleave NP groups from BNPP (see Results).

The kinetic analysis was carried out in two ways. First, taking into account the production of two NP ions, the data were fitted to the expression $A = B + C \exp(-k_{obs1}t) + D \exp(-k_{obs2}t)$ to determine the rate constants (k_{obs1} and k_{obs2}) for the release of each NP. For some reactions, typically slower reactions such as those carried out at low pH, instrumental drift led to poor fits to the data. Consequently, initial rates were determined by the method applied previously by others (for example, see ref 54) and converted into observed rate constants (k_{obs1}). Second-order rate constants for the release of the first NP anion were determined from the two sets of k_{obs1} by fitting the data to eq 7 derived from Scheme 2 (see Results).

(iii) Catalytic Turnover. A set of spectrophotometric screening experiments were carried out at pH 9.1, [BNPP] = 1 mM, $T = 50$ °C, and [complex] = 1−100 μM. This was followed by a more comprehensive series of experiments conducted under the following conditions: Solutions of BNPP (1 mM), $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ (100, 25, 10, and 1 μM), and $NaClO_4$ (to maintain $I = 0.15$ M) in 50 mM of buffer (MOPS, pH 5.9; MES, pH 7.0; CHES, pH 9.1), as well as a control for each pH, identical to the other solutions but containing no copper(II) complex, were prepared and incubated in an oven at 50 °C for 63 days. Samples were removed at intervals, and the electronic spectra were measured in the 200−1200 nm range at 50 °C. The aliquots were returned to the stock solution. In the cases where the absorbance of the aliquot exceeded the limits of the instrument, the aliquot was diluted prior to analysis and discarded after measurement. The absorbance at 400 nm was corrected for the uncatalyzed BNPP hydrolysis at each pH by subtracting the absorbance of a control experiment. NP concentrations, and hence turnover numbers, were then determined using molar extinction coefficients for NP of 1600, 11800, and 20000 M^{-1} cm^{-1} at pH 5.9, 7.4, and 9.1, respectively.

Supporting Information Available: Tables of observed rate constants k_{obs1} determined at various pH's, complex concentrations, and temperatures (Tables S1 and S2); figure showing the biphasic nature of the cleavage of BNPP by $[Cu(Me_3tacn)(OH_2)_2]^{2+}$ (Figure S1); figure showing the fitting of the data to eq 7 (Figure S2); and ^{31}P NMR spectra showing the conversion of BNPP into NPP and NPP into phosphate (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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