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Rapid Phosphodiester Hydrolysis by an Ammonium-Functionalized Copper(II) Complex. A Model for the Cooperativity of Metal Ions and NH-Acidic Groups in Phosphoryl Transfer Enzymes

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Abstract: The copper(II) complexes $[(\mathbf{L^n})Cu(NO_3)_4{\cdot}2(H_2O)]$ ($n = 1$: **1**, $n = 2$: **2**) of the ammonium-functionalized ligands $[6,6'\text{-}(Me_2HNCH_2C{\equiv}C)_2bpy]^{2+}$ ($\mathbf{L^1}$) and $[6,6'\text{-}(Me_3NCH_2C{\equiv}C)_2bpy]^{2+}$ ($\mathbf{L^2}$) were prepared. Hydrolysis of the activated phosphodiester bis(*p*-nitrophenyl) phosphate (BNPP) by these complexes in ethanol−water 19:1 at 20 °C was investigated. The rate constants for cleavage of the bound phosphodiester at pH 6.6 are $k_{cat} = 4.4(\pm 0.4) \times 10^{-3}\ s^{-1}$ for $(\mathbf{L^1})Cu$ and $k_{cat} = 4(\pm 1) \times 10^{-6}\ s^{-1}$ for $(\mathbf{L^2})Cu$. $(\mathbf{L^1})Cu$ accelerates hydrolysis of BNPP 4×10^7-fold and is 1000 times more reactive than $(\mathbf{L^2})Cu$. This suggests that the high reactivity of $(\mathbf{L^1})Cu$ is related to the interaction of the acidic $-NMe_2H^+$ group with the phosphodiester substrate. Bifunctional binding of a phosphate ester by metal coordination and hydrogen bonding with one ammonium group is observed in the crystallographically characterized complex $[(\mathbf{L^1})_2Cu_2(1,3\text{-}\mu\text{-}O_3POPh)_2(OH_2)_2](NO_3)_4$ (**3**). A plausible mechanism of BNPP cleavage by $(\mathbf{L^1})Cu$ includes metal-hydroxide attack to the phosphodiester which is doubly activated by coordinative and hydrogen bonding. The copper(II) complex of $\mathbf{L^1}$ represents a simple model for the efficient cooperativity of metal ions and NH-acidic amino acid side chains (Lys-ammonium, Arg-guanidinium, His-imidazolium) in enzymes that catalyze the cleavage of phosphate di- and monoesters.

Introduction

Understanding the basic principles of enzymatic phosphoryl transfer reactions is fundamental for the rational design of efficient synthetic hydrolysis catalysts. A particular attractive goal is the development of artificial phosphodiesterases for the nonoxidative cleavage of the phosphodiester backbone in nucleic acids. Such reagents might have important applications in future biotechnology.[1]

In many metal dependent phosphoryl transfer enzymes catalysis is supported by amino acid side chains which act as acids, bases or nucleophiles. The NH-acidic groups are represented by the positively charged residues Arg-guanidinium, His-imidazolium and Lys-ammonium. These groups stabilize the pentacoordinate transition state (intermediate, respectively) of the nucleophilic substitution reaction at phosphorus by hydrogen bonding or proton transfer. In staphylococcal nuclease Ca^{2+} and Arg-guanidinium electrostatically activate the substrate and facilitate attack of a nucleophile, possibly a calcium-coordinated hydroxide.[2] Active site cooperativity of Zn^{2+} and Lys-ammonium was proposed for phosphodiester hydrolysis by nuclease S1 from *Aspergillus orycae*.[3] Metal ion promoted phosphate monoester hydrolysis is supported by Arg-guanidinium or His-imidazolium residues in alkaline phosphatase from *E. coli*,[4] purple acid phosphatase from kidney beans[5] and in calcineurin.[6] The tetrahedral transition state of metal-mediated peptide bond cleavage is stabilized by a Lys-ammonium group in bovine lens aminopeptidase.[7]

Quite recently, several interesting studies have demonstrated that the reactivity of simple metal complexes toward phosphodiesters can be increased substantially by attachment of basic or nucleophilic auxiliary groups to the ligand.[8] In contrast, the highly efficient cooperativity of a metal ion and a NH-acidic group has not yet been succesfully mimicked with a simple model system.[9] For this purpose we have designed the ammonium-substituted 2,2′-bipyridine (bpy) ligand $\mathbf{L^1}$. The zinc(II) complex of this ligand is capable of cleaving bis(*p*-

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nitrophenyl) phosphate (BNPP), but it is only about 5 times more reactive than related zinc complexes that do not carry functional groups.[10] A preliminary study[11] revealed that the copper(II) compound of $\mathbf{L^1}$ has a much higher reactivity than ($\mathbf{L^1}$)Zn. Although copper(II) is not a natural cofactor of phosphodiesterase enzymes, various studies on the hydrolysis of activated phosphodiesters and of RNA by copper(II) complexes have contributed to the general understanding of metal promoted phosphodiester hydrolysis.[12,8d]

In this paper we present a detailed kinetic study of BNPP hydrolysis by ($\mathbf{L^1}$)Cu together with crystallographic evidence for bifunctional interaction of the complex with a phosphate ester.

Experimental Section

General Information. All reagents unless otherwise indicated were of analytical grade and were used without further purification. Ethanol−water solvent mixtures were prepared from absolute ethanol and ultrapure water. 2,4,6-Trimethylpyridine was distilled before use. pH measurements in ethanol−water 19:1 (v/v) were performed with a combined glass electrode calibrated with aqueous buffers and were reproducible within ±0.05 units. The pH values given in this work correspond to pH meter reading + 0.8. This correction value was obtained by extrapolation of literature data for ethanol−water mixtures.[13] EPR spectra were recorded on a Bruker ESP 300 X-band spectrometer using ethanol−water 19:1 glasses at 77 K. UV-vis spectra were measured on a Shimadzu UV 3100 spectrometer.

6,6′-Bis(3-dimethylaminopropynyl)-2,2′-bipyridine ($\mathbf{L^1}$-2 H) and $\mathbf{L^1}(NO_3)_2$ were prepared by previously reported methods.[10]

6,6′-Bis(3-trimethylammoniopropynyl)-2,2′-bipyridine dinitrate ($\mathbf{L^2}(NO_3)_2$). 6,6′-Bis(3-dimethylaminopropynyl)-2,2′-bipyridine[10] (200 mg, 0.63 mmol) was dissolved in 50 mL of acetonitrile. A solution containing CH_3I (0.39 mL, 6.3 mmol) in 5 mL of acetonitrile was added dropwise with stirring. After 15 min the white precipitate was centrifuged off and washed with 20 mL of acetonitrile. The product was suspended in 50 mL of ethanol, and 12.6 mL of a 0.10 M aqueous $AgNO_3$ solution was added with stirring. After 0.5 h the precipitate was removed by filtration and washed with 20 mL of ethanol. Filtrate and washing solution were combined and reduced to dryness *in vacuo*. The white residue was dried in vacuo for 5 h (172 mg, yield 58%). ^1H NMR (D_2O, 300 MHz): δ 3.51 (18 H, s, CH_3), 4.76 (4 H, s, CH_2), 7.98 (2 H, d, $J = 7.6$ Hz, bpy-H), 8.26 (2 H, t, $J = 7.9$ Hz, bpy-H), 8.33 (2 H, d, $J = 8.1$ Hz, bpy-H). UV (ethanol−water 19:1): $\lambda_{max} = 297$ nm ($\epsilon = 1.76 \times 10^4$ M^{-1} cm^{-1}). Anal. Calcd for $C_{22}H_{28}N_6O_6$ (472.5): C, 55.92; H, 5.97; N, 17.78. Found: C, 55.30; H, 6.11; N, 18.20.

[($\mathbf{L^1}$)Cu(NO_3)$_4$·2(H_2O)] (1). $\mathbf{L^1}(NO_3)_2$ (22 mg, 50 μmol) was dissolved in a mixture of 250 μL of water and 3.6 mL of dry ethanol. A stock solution (1.18 mL) of $Cu(NO_3)_2 \cdot 3H_2O$ (40 mM in ethanol) was added with stirring. The green solution was allowed to stand at room temperature for 2 h. Green crystals of **1** were isolated and air dried (yield 19 mg, 56%). UV (ethanol−water 19:1): $\lambda_{max} = 322$ nm ($\epsilon = 1.21 \times 10^4$ M^{-1} cm^{-1}), 333 ($\epsilon = 1.27 \times 10^4$). Anal. Calcd for $C_{20}H_{28}CuN_8O_{14}$ (668.0): C, 35.91; H, 4.36; N, 16.75. Found C, 35.67; H, 4.32; N, 16.18.

[($\mathbf{L^2}$)Cu(NO_3)$_4$·2(H_2O)] (2). $\mathbf{L^2}(NO_3)_2$ (23 mg, 49 μmol) was dissolved in 250 μL of water. Five hundred microliters of a $Cu(NO_3)_2 \cdot 3H_2O$ stock solution (97 mM in ethanol) and 4.2 mL of dry ethanol were added subsequently with stirring. Cooling to 5 °C for 1 h yielded green crystals which are isolated and air dried (yield 11 mg, 32%). UV (ethanol−water 19:1): $\lambda_{max} = 321$ nm ($\epsilon = 1.26 \times 10^4$ M^{-1} cm^{-1}), 333 ($\epsilon = 1.30 \times 10^4$). Anal. Calcd for $C_{22}H_{32}CuN_8O_{14}$ (696.1): C, 37.96; H, 4.63; N, 16.10. Found C, 37.79; H, 4.51; N, 15.80.

[($\mathbf{L^1}$)$_2$Cu$_2$(1,3-μ-O$_3$POPh)$_2$(OH$_2$)$_2$](NO$_3$)$_4$·2 EtOH·2H$_2$O (3). To a solution of $\mathbf{L^1}(NO_3)_2$ (22 mg, 50 μmol) and disodium phenyl phosphate (15 mg, 50 μmol) in 3 mL of ethanol−water (19:1) was added with stirring $Cu(NO_3)_2 \cdot 3H_2O$ (12 mg, 50 μmol). The solution was allowed to stand for 2 h. Bright blue X-ray quality crystals were obtained. The solvent was decanted, and the product was washed with few diethyl ether and air dried (yield 19.6 mg, 52%). Anal. Calcd for $C_{56}H_{78}Cu_2N_{12}O_{26}P_2$ (1524.3): C, 44.16; H, 5.16; N, 11.03. Found C, 43.21; H, 4.85; N, 10.95.

Spectrophotometric Titration. A solution of the ligand ($\mathbf{L^1}(NO_3)_2$ or $\mathbf{L^2}(NO_3)_2$, respectively, 50 μM) and sodium diphenyl phosphate (100 mM) was prepared in ethanol−water 19:1 (v/v). The pH of the solution was adjusted to 6.6 with NaOH in ethanol−water 19:1. Appropriate amounts of a $Cu(NO_3)_2 \cdot 3H_2O$ stock solution (5 mM in ethanol−water 19:1) were added with stirring. Immediately after addition of metal salt a UV spectrum of the solution was taken at 20 °C, and the pH was readjusted to 6.6 when necessary. The absorbance at 333 nm was corrected for the weak absorbance of Cu^{2+} ions in the absence of ligand under the same conditions ($\epsilon_{333} = 3.6 \times 10^2$ M^{-1} cm^{-1}). An isosbestic point at ca. 313 nm was found when data were corrected for the absorbance of excess Cu ($\epsilon_{313} = 7.0 \times 10^2$ M^{-1} cm^{-1}). Stability constants were calculated by fitting the increase in 333 nm absorbance for Cu:L ratios > 1 to a 1:1 complex formation model, using $\epsilon_{333} = 1.27 \times 10^4$ for ($\mathbf{L^1}$)Cu and $\epsilon_{333} = 1.30 \times 10^4$ for ($\mathbf{L^2}$)Cu (the free ligands do not absorb at this wavelength).

pH Titration. A solution of $\mathbf{L^1}(NO_3)_2$ or **1** (5 mM), respectively, containing sodium diphenyl phosphate (100 mM), was prepared in ethanol−water 19:1 (v/v). Aliquots (50 μL) of a CO_2-free NaOH solution (50 mM) in ethanol−water 19:1 were added with stirring. The pH was measured with a glass electrode and read after 30 s equilibration time.

Kinetics. Reaction solutions were prepared by combining appropriate amounts of ligand ($\mathbf{L^1}(NO_3)_2$, $\mathbf{L^2}(NO_3)_2$, or bpy), $Cu(NO_3)_2 \cdot 3H_2O$ (stock solution in ethanol), 2,4,6-trimethylpyridinium bis(*p*-nitrophenyl)phosphate (200 mM stock solution in ethanol, 1:1 mixture of 2,4,6-trimethylpyridine and bis(*p*-nitrophenyl)phosphoric acid) and 2,4,6-trimethylpyridinium nitrate. The final composition of solvent mixture was ethanol−water 19:1 (v/v). It was not possible to perform the kinetic experiments in the presence of excess (>0.3 M) standard electrolytes (e.g., $NaClO_4$, $NaNO_3$) owing to precipitate formation. To achieve a constant ionic strength ($I \approx 0.1$ M) the 2,4,6-trimethylpyridinium salts of bis(*p*-nitrophenyl)phosphate and nitrate, respectively, were combined such that the total salt concentration was 100 mM in all reaction solutions. The reaction was initiated by adjusting the desired pH by addition of NaOH solution in ethanol−water 19:1. This does not result in a change of ionic strength since the 2,4,6-trimethylpyridinium cation is replaced by Na^+. For the pH-rate-profiles shown for ($\mathbf{L^1}$)Cu and ($\mathbf{L^2}$)Cu (Figure 6) the Na^+/2,4,6-trimethylpyridinium ratio increases with increasing pH. In a control experiment 2,4,6-trimethylpyridine was used instead of NaOH to adjust pH 6.3; the same hydrolysis rates were determined. This observation rules out that reactivity is significantly affected by medium effects. In the absence of buffer, initial rates appeared to be similar to those in the buffered reaction solutions, but accurate data were difficult to obtain since the rate readily decreases owing to a decrease in pH.

At pH < 6.6 in ethanol−water 19:1 (v/v) *p*-nitrophenol does in effect not dissociate into *p*-nitrophenolate and protons. To detect the increase in *p*-nitrophenol concentration in the reaction solutions (10 mL), at least five 0.1 ml samples were taken in appropriate time intervals and mixed with 3 mL of 40 mM NaOH solution in ethanol−water (2:3)

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containing 2 mM EDTA. The produced *p*-nitrophenolate ($\epsilon_{400} = 19\ 100$ $M^{-1}\ cm^{-1}$ in ethanol−water (2:3)) was detected photometrically. For <2% conversion of BNPP an approximately linear increase of *p*-nitrophenolate concentration with time was observed in all cases (correlation coefficients > 0.99). Control experiments in which BNPP was replaced by diphenyl phosphate show that the complexes themselves do not develop undesirable absorbances at 400 nm in course of the kinetic experiments.

To characterize reaction products of BNPP hydrolysis selected solutions were treated with EDTA (2 equiv per equiv Cu) and reduced to dryness. The residue was taken up in a minimum amount of ethanol−water (1:1). ^{31}P NMR spectra displayed signals for unreacted BNPP (−11.2 ppm vs external H_3PO_4) and for the monoester nitrophenyl phosphate (1.7 ppm) but not for phosphate (expected at 6.8 ppm).

To determine the spontaneous hydrolysis of BNPP a 100 mM solution of 2,4,6-trimethylpyridinium bis(*p*-nitrophenyl)phosphate was prepared in ethanol−water 19:1 and adjusted to pH 6.6 as described above. The kinetic measurements were performed at 20(±0.5) °C. All data are average values of three kinetic runs.

Reaction solutions containing ($\mathbf{L}^1$)Cu and ($\mathbf{L}^2$)Cu turned reddish brown on prolonged standing. This is attributed to an intense 485 nm CT-band of $[(\mathbf{L})_2Cu]^+$ complexes formed by Cu(II)−Cu(I) reduction. It was estimated that in the kinetic experiments <5% of the initially present Cu(II) complex is reduced. A 1:1 mixture of $\mathbf{L}^1$ and copper(I) perchlorate is not reactive toward BNPP. Furthermore, we can rule out that the high reactivity of ($\mathbf{L}^1$)Cu is related to the cooperativity of copper(II) and peroxide which eventually might form by reaction of Cu(I) with dioxygen. The same rate enhancements are observed when the kinetic experiments are run on air or strictly under oxygen free conditions. It was reported that cleavage of amides and phosphodiesters is rapidly promoted by metal-peroxide cooperativity.[14]

X-ray Diffraction Studies. X-ray quality crystals of $[(\mathbf{L}^1)_2Cu_2(1,3\text{-}\mu\text{-}O_3POPh)_2(OH_2)_2](NO_3)_4{\cdot}2EtOH{\cdot}2H_2O$ (**3**) were obtained during the preparation of the complex. An appropriate single crystal was fixed on a glass fiber with grease and cooled to 170 K.

X-ray data were collected on a Siemens P3 diffractometer using MoKα-radiation (0.71073 Å). The structure was solved using direct methods (SHELXS-86)[15] and refined by full-matrix least-squares against F^2 (SHELXL 93).[16] Anisotropic thermal parameters were used for all non-hydrogen atoms. H(N3) and H(O1) were localized in the final difference Fourier map and were refined with variable U_{iso}. Other hydrogen atom parameters follow a riding model. The molecular sites of solvate water and ethanol and of one nitrate counter ion are partially occupied. During refinement the positional occupancies were fixed at a value of 0.5.

Formula $C_{56}H_{78}Cu_2N_{12}O_{26}P_2$, formula weight 1524.3, crystal system triclinic, space group $P\bar{1}$; light blue single crystal with dimensions 0.1 × 0.2 × 0.15 mm; $a = 11.718(2)$, $b = 12.121(2)$, $c = 13.037(3)$ Å, $\alpha = 74.90(3)$, $\beta = 85.40(3)$, $\gamma = 75.25(3)°$, $V = 1728.7(6)$ Å^3, $Z = 1$; ω-scan, no absorption correction, $4° < 2\theta < 54°$, 7949 reflections measured, 7575 independent, 5493 observed with $I > 2\sigma(I)$, 469 parameters; R (on F) = 0.0496, wR (on F^2) = 0.1417, GOF 0.749; max./min. residual electron density: 1.507/−0.47 e Å^{-3} (the maximum is localized at a disordered nitrate counterion).

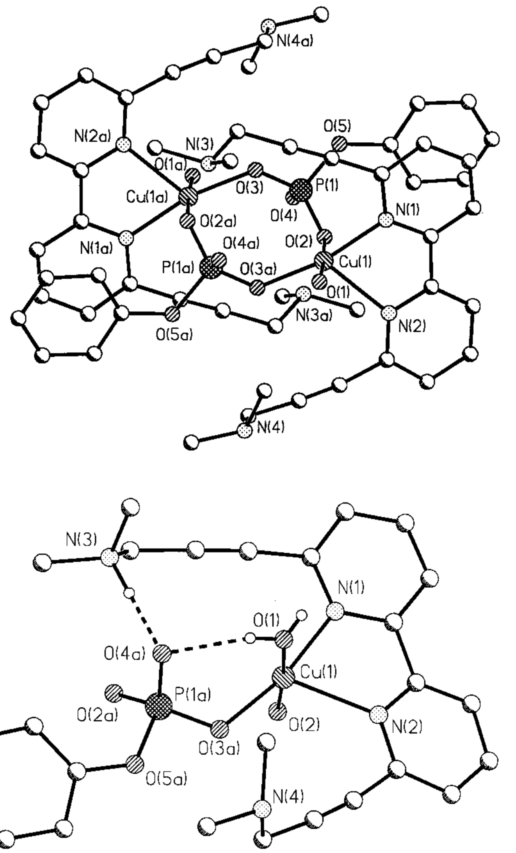

Figure 1. Crystal structure of $[Cu_2(\mathbf{1})_2(1,3\text{-}\mu\text{-}PO_3OPh)_2(OH_2)_2]^{4+}$ (top) and of the mononuclear $[Cu(\mathbf{1})(1,3\text{-}\mu\text{-}PO_3OPh)(OH_2)]$ subunit of the centrosymmetric complex. Hydrogen atoms at N(3) and O(1) were located in the difference Fourier map and refined with variable U_{iso}. Other H-atoms are omitted for clarity. Selected bond distances [Å] and angles [deg]: Cu(1)−O(1) 1.982(3), Cu(1)−O(2) 1.937(2), Cu(1)−O(3a) 1.932(2), Cu(1)−N(1) 2.021(3), Cu(1)−N(2) 2.273(3); O(1)−Cu(1)−O(2) 175.8(1), O(3a)−Cu(1)−N(1) 167.5(1), O(3a)−Cu(1)−O(1) 88.5(1), O(3a)−Cu(1)−O(2) 90.1(1), N(1)−Cu(1)−N(2) 76.2(1); N(3)−O(4a) 2.666(5), O(1)−O(4a) 2.735(4), N(3)−H−O(4a) 176(5), O(1)−H−O(4a) 153(5).

Results and Discussion

We have communicated a preliminary kinetic study on hydrolysis of the activated phosphodiester BNPP by the copper(II) complex of $\mathbf{L}^1$.[11] The *p*-nitrophenyl substrate was used since unactivated phosphodiesters are highly resistant to hydrolysis.[17] $\mathbf{L}^1$ carries two $-NMe_2H^+$ group linked to a metal binding 2,2′-bipyridine (bpy) unit by alkyne spacers. In the complexes the pendant ammonium groups are perfectly positioned to form hydrogen bonds with a phosphodiester coordinated to the metal. At the same time, the spacers prevent intramolecular metal chelation by $-NMe_2$.

Structure of Complexes. The green crystalline complexes $[(\mathbf{L}^1)Cu(NO_3)_4{\cdot}2H_2O]$ (**1**) and $[(\mathbf{L}^2)Cu(NO_3)_4{\cdot}2H_2O]$ (**2**) were obtained from the ligands and $Cu(NO_3)_2{\cdot}3H_2O$ in ethanol−water 19:1. Reaction of $\mathbf{L}^1(NO_3)_2$ with $Cu(NO_3)_2{\cdot}3H_2O$ and the monoester disodium phenyl phosphate yielded bright blue crystals of the dimeric complex $[(\mathbf{L}^1)_2Cu_2(1,3\text{-}\mu\text{-}O_3POPh)_2(OH_2)_2](NO_3)_4{\cdot}2EtOH{\cdot}2\ H_2O$ (**3**) which has a crystallographic centre of symmetry (Figure 1). The copper ions are bridged by two phenyl phosphate molecules. Each copper is coordinated by the bpy-unit of $\mathbf{L}^1$, two phosphate oxygens and one water molecule. The N_2O_3 coordination polyhedron is a square based pyramid. Four short bonds are formed in the basal plane with O(1), O(2), O(3a) and N(1). The bond with the second bpy nitrogen N(2) in the apical position is significantly longer. This

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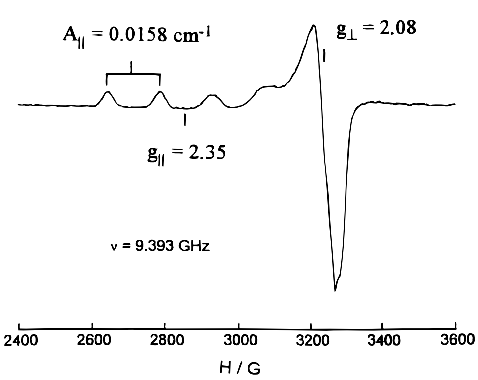

Figure 2. EPR spectrum of a glassy ethanol−water 19:1 solution at 77 K, containing **1** (5 mM), diphenyl phosphate (70 mM), and 2,4,6-trimethylpyridine buffer (100 mM) at pH 6.3.

particular coordination geometry has also been observed in **1**[18] and in [{6,6′-(pentyne-1-yl)$_2$bpy}$_2$Cu$_2$(μ-Cl)$_2$(Cl)$_2$][19] and appears to be a consequence of the steric influence of the alkyne substituents. In copper(II) complexes of 2,2′-bipyridine usually both Cu−N$_{bpy}$ bonds are short in-plane interactions. Thus, the related complex [(bpy)$_2$Cu$_2$(1,3-μ-O$_3$POR)$_2$(OH$_2$)$_2$](NO$_3$)$_2$ (R = adenosyl) displays a square-pyramidal geometry with in-plane O$_2$N$_2$ coordination and the apical position occupied by a water molecule.[20]

Another interesting feature of the structure in Figure 1 is hydrogen bonding of the phosphate monoester with one ammonium group, as indicated by the interatomic distance N(3)−O(4a) of 2.67 Å. This clearly shows that the (**L^1**)Cu fragment can provide double electrostatic activation of a phosphate ester by both metal coordination and hydrogen bonding. A second hydrogen bond is formed between O(4a) and coordinated water, O(4a)−O(1) being 2.74 Å. O(1) is located only 3.2 Å away from the phosphorus atom. A metal hydroxide which is generated by deprotonation of coordinated water might well act as a nucleophile and attack the phosphorus atom of the coordinated substrate.

Information on the copper coordination geometry in an ethanol−water 19:1 glass is obtained from EPR spectroscopy. EPR solutions had the same composition as those used for kinetic experiments (see below), but BNPP was replaced by the less reactive diphenyl phosphate (DPP) (Figure 2). The spectrum is typical for Cu(II) complexes with approximate tetragonal symmetry, and according to empirical plots of Peisach and Blumberg the values $g_{\parallel}$ = 2.35 and $A_{\parallel}$ = 0.0158 cm^{-1} are in agreement with an in-plane O$_3$N donor set.[21] Thus, the coordination geometry found in the crystalline state appears to be retained in glassy solution. A very similar EPR spectrum is obtained for (**L^2**)Cu under the same conditions, $g_{\parallel}$ = 2.36 and $A_{\parallel}$ = 0.0156 cm^{-1}. In contrast, the literature EPR parameters for [(bpy)Cu(OH$_2$)$_2$]$^{2+}$ with equatorial N$_2$O$_2$-coordination, $g_{\parallel}$ = 2.29−2.31 and $A_{\parallel}$ = 0.0166−0.0169 cm^{-1}, are significantly different.[22] EPR spectra show that in 1:1 metal-to-ligand mixtures (**L^1**)Cu and (**L^2**)Cu are the major species. Signal patterns for (**L**)$_2$Cu and for Cu^{2+} ions are not detectable.

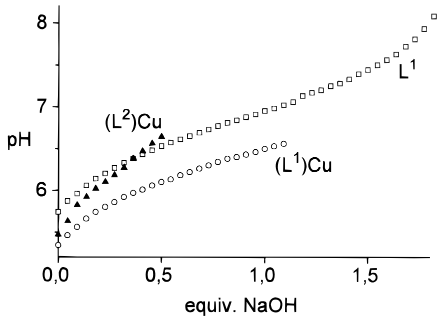

Figure 3. Titration of **L^1**, **1**, and **2** with NaOH. Conditions: ethanol−water 19:1, [sodium diphenyl phosphate] = 100 mM, T = 20(±0.5) °C.

Complex Formation in Solution. Whereas bpy forms a rather stable 1:1 complex with Cu^{2+} in aqueous solution (formation constant $K = 10^{6.3}$ M^{-1} at 25 °C),[23] the copper complexes of **L^1** and **L^2** have surprisingly low stability in water. To achieve complex formation an ethanol−water 19:1 solvent mixture was used for the kinetic experiments.

Formation constants of the 1:1 complexes in this medium were obtained by spectrophotometric titration of **L** with copper(II) nitrate in the presence of DPP. The latter phosphodiester is much less reactive than BNPP and is not hydrolyzed during the manipulations.

Coordination of copper to the bpy moiety of the ligands is easily monitored by the shift of the bpy $\pi-\pi^*$ band from $\lambda_{max} \approx$ 298 nm in the free ligands to λ_{max} = 333 nm in the complexes. For Cu:L ratios > 1 an isosbestic point at 313 nm was observed, and the increase in 333 nm absorbance could be fitted to a 1:1 model. Equilibrium constants $K = 1.8(\pm0.1) \times 10^4$ M^{-1} for the formation of (**L^1**)Cu from **L^1** and Cu^{2+} and $K = 2.1(\pm0.1) \times 10^4$ M^{-1} for the formation (**L^2**)Cu from **L^2** and Cu^{2+} were calculated (pH 6.6, 20 °C and 100 mM DPP concentration).

pH Titration. Ethanol−water 19:1 solutions of **L^1**(NO$_3$)$_2$ and of complexes **1** and **2** in the presence of 100 mM sodium diphenyl phosphate were titrated with NaOH (Figure 3). Titration of the copper complexes was limited to pH < 6.8 because of precipitate formation at higher pH. The limited data available for the complexes and the low accuracy of pH measurements in ethanol−water 19:1 did not allow a detailed analysis of the titration curves. We have estimated protonation constants using the equation pK_a = pH + log ([acid]/[base]) which is applicable to pK_a determination of weak acids.[13b] For the ammonium groups of **L^1**(NO$_3$)$_2$ pK_a = 7.0 is derived from the pH after addition of 1.0 equiv of NaOH. For **2** pK_a = 6.6 is estimated for Cu-coordinated water from the pH at 50% neutralization. This value should correspond to the acidity constant of H$_2$O in (**L^2**)Cu(OH$_2$)[O$_2$P(OR)$_2$] since kinetic data (see below) indicate substrate saturation at 100 mM phosphodiester concentration. (**L^1**)Cu is expected to have three titratable protons (two −NMe$_2$H$^+$ and Cu-coordinated H$_2$O). If we speculate that all three acidic groups have the same pK_a a best fit of experimental and theoretical pH values at 20, 27 and 33% neutralization (addition of 0.6, 0.8 and 1.0 equiv NaOH) is obtained for p$K_a \approx$ 7.0.

Kinetics. Reactivities of (**L^1**)Cu, (**L^2**)Cu and (bpy)Cu toward the activated phosphodiester BNPP were compared. (**L^1**)Cu and (**L^2**)Cu were used in 5 mM concentration. Extrapolation of the stability data obtained by spectrophotometric titration reveals that under these conditions < 10% dissociation of the complexes into free ligand and metal ion is expected. Initial rates (<2%

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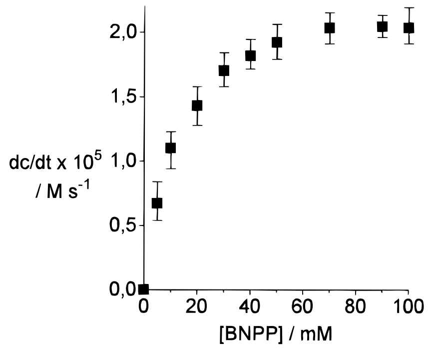


Figure 4. Saturation kinetics for the hydrolysis BNPP by ($\mathbf{L^1}$)Cu^{4+} (5 mM) in ethanol−water 19:1. Conditions: pH 6.6(±0.1), 20(±0.5) °C, $I \approx 100$ mM, buffer: 2,4,6-trimethylpyridine (100 mM).

conversion of BNPP) were determined by spectrophotometric determination of the released *p*-nitrophenolate. By ^{31}P NMR analysis of the reaction solutions only signals of the monoester nitrophenyl phosphate but not for phosphate could be detected.

In a previous communication[11] we have shown that BNPP cleavage rate is linearly dependent on ($\mathbf{L^1}$)Cu concentration. Dependence of hydrolysis rate on the concentration of BNPP is shown in Figure 4. Saturation kinetics with good Michaelis−Menten behaviour is observed at pH 6.6. A plot of $1/v$ vs $1/c$ gave $k_{cat} = 4.4 \times 10^{-3}$ s^{-1} and $K_M = 0.013$ M. BNPP bound to ($\mathbf{L^1}$)Cu hydrolyzes 4×10^7 times faster than free BNPP [$k_{uncat} = 1.1 \times 10^{-10}$ s^{-1}] under the same conditions (Table 1). $\mathbf{L^1}$ in the absence of copper has no significant effect on BNPP hydrolysis.

For ($\mathbf{L^2}$)Cu kinetic measurements were complicated by progressive Cu(II)-Cu(I) reduction on prolonged reaction times. Reliable kinetic data were obtained for pH > 6.3 and for high BNPP concentrations. Initial reaction rates (dc/dt) in the presence of 2.5 mM ($\mathbf{L^2}$)Cu and 5 mM ($\mathbf{L^2}$)Cu were $0.9(\pm 0.2) \times 10^{-8}$ and $2.0(\pm 0.5) \times 10^{-8}$ M s^{-1}, respectively, at pH 6.6, 20 °C and 100 mM BNPP concentration. This is indicative of a first order dependence of rate on ($\mathbf{L^2}$)Cu concentration (at lower complex concentration data were not well reproducible, and precipitates formed at higher concentration of ($\mathbf{L^2}$)Cu). No further increase of rate was observed when the BNPP concentration was increased from 70 to 100 mM, indicating substrate saturation of the complex. Thus, $k_{cat} = 4 \times 10^{-6}$ s^{-1} for ($\mathbf{L^2}$)Cu at pH 6.6 was derived directly from v_{max}.

Trogler and Morrow previously described a detailed kinetic study on the hydrolysis of BNPP and the related substrate *p*-nitrophenyl ethyl phosphate by (bpy)Cu in aqueous solution at 75 °C.[12a] These data are not directly comparable to our results in ethanol−water 19:1. Therefore, we have reinvestigated BNPP cleavage by (bpy)Cu in the ethanolic medium at 20 °C. Rate is linearly dependent on complex concentration for 0.1 mM < [(bpy)Cu^{2+}] < 1 mM. A plot of rate *vs* BNPP concentration at pH 6.7 and 1 mM (bpy)Cu is shown in Figure 5; Michaelis−Menten analysis of these data gave $k_{cat} = 1.5 \times 10^{-6}$ s^{-1} and $K_M = 0.0083$ M. The smaller value of K_M for (bpy)Cu compared to ($\mathbf{L^1}$)Cu indicates that BNPP has a higher affinity to the former complex since K_M^{-1} corresponds to the substrate binding constant of the complexes. Possibly this is a consequence of some steric hindrance by the 6,6′-substituents in ($\mathbf{L^1}$)Cu.

A comparison of the complexes based on k_{cat} reveals that ($\mathbf{L^1}$)Cu is 1000 times more reactive toward BNPP than ($\mathbf{L^2}$)Cu and 2900 times more reactive than (bpy)Cu under the same conditions (Table 1).

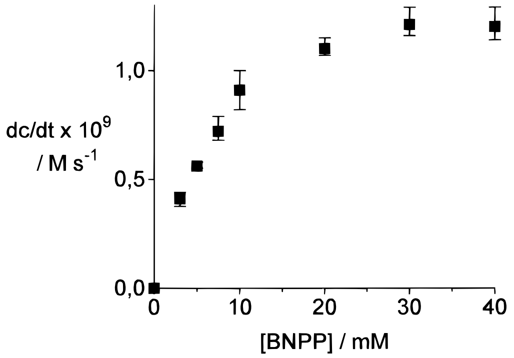


Figure 5. Saturation kinetics for the hydrolysis BNPP by (bpy)Cu^{2+} (1 mM) in ethanol−water 19:1. Conditions: pH 6.6(±0.1), 20(±0.5) °C, $I \approx 100$ mM, buffer: 2,4,6-trimethylpyridine (100 mM).

Table 1. Observed and Relative Values of k_{cat} for BNPP Hydrolysis by Copper(II) Complexes[a]

catalyst	k_{cat} (s^{-1})	k_{rel}
($\mathbf{L^1}$)Cu	$4.4(\pm 0.4) \times 10^{-3}$	4×10^7
($\mathbf{L^2}$)Cu	$4(\pm 1) \times 10^{-6}$	4×10^4
(bpy)Cu	$1.5(\pm 0.2) \times 10^{-6}$	1.4×10^4
$\mathbf{L^1}$	$1.2(\pm 0.3) \times 10^{-10}$	1.1
none (k_{uncat})	$1.1(\pm 0.3) \times 10^{-10}$	1

[a] Error limits are based on the reproducibility of kinetic measurements. For reaction conditions see Figure 5.

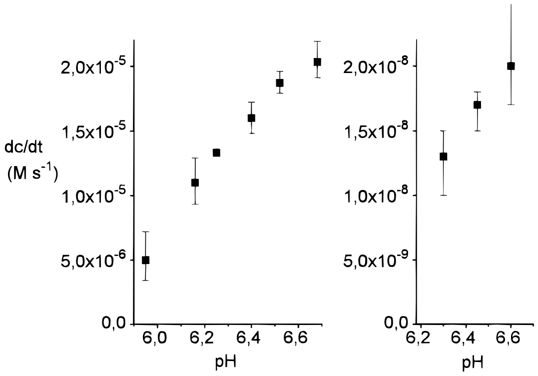


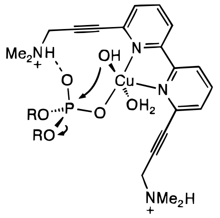
Figure 6. Dependence of BNPP hydrolysis by ($\mathbf{L^1}$)Cu^{4+} and ($\mathbf{L^2}$)Cu^{4+} (5 mM) on pH. Conditions: ethanol−water 19:1, [BNPP] = 100 mM, 20(±0.5) °C, $I \approx 100$ mM, buffer: 2,4,6-trimethylpyridine (100 mM).

($\mathbf{L^1}$)Cu and ($\mathbf{L^2}$)Cu display a similar increase of rate with pH (Figure 6). Provided that Cu-OH is the reactive nucleophile, $pK_a \approx 6.8$ of coordinated water can be roughly estimated for both complexes from the kinetic data. For ($\mathbf{L^2}$)Cu this is in reasonable agreement with the value obtained by pH titration, $pK_a \approx 6.6$. The pH-rate profiles show that the very different reactivities of ($\mathbf{L^1}$)Cu and ($\mathbf{L^2}$)Cu at pH 6.6 cannot be attributed to large differences in the pK_a of copper coordinated water. At higher pH values one would expect for ($\mathbf{L^1}$)Cu a decrease of reactivity and a bell shaped pH-rate profile owing to deprotonation of the ammonium groups. However, this effect is not yet significant for pH < 6.6. We believe that only one of the two ammonium groups is involved in catalysis. Statistically, at pH 6.6 at least one ammonium group is protonated in 92% of the ($\mathbf{L^1}$)Cu molecules if both ammonium groups have $pK_a = 7.0$.

In case of (bpy)Cu rate increases with pH and reaches a plateau at pH ≈ 6.7. At pH > 7.2 reactivity decreases significantly. Trogler and Morrow[12a] attributed a decrease of (bpy)Cu activity in alkaline aqueous solution to the formation of inactive hydroxide-bridged dimers (bpy)Cu(μ-OH)$_2$Cu(bpy).

The reactivity of ($\mathbf{L^1}$)Cu toward BNPP decreases more than 10-fold when the water content of the ethanol−water mixture is increased from 5% to 15%. This cannot be attributed

Chart 1. Plausible Mechanism of BNPP Hydrolysis by (**L^1**)Cu (R = *p*-nitrophenyl).



exclusively to enhanced dissociation of the complex into free ligand and metal ion, as observed by spectrophotometry. Rather, interaction of $-NMe_2H^+$ with the phosphodiester substrate is disfavored by H_2O which is a highly competitive solvent for hydrogen bonding interactions.[24]

The Substrate ApA. Linkletter and Chin have discovered that (2,9-dimethyl-1,10-*o*-phenanthroline)Cu^{2+} rapidly cleaves the RNA dinucleotide ApA in aqueous solution.[12e] Interestingly, in crystals of [(2,9-dimethyl-1,10-*o*-phenanthroline)$_2$Cu$_2$(μ-Cl)$_2$-(Cl)$_2$][25] and [(**L^1**)$_2$Cu$_2$(1,3-μ-O$_3$POPh)$_2$(OH$_2$)$_2$](NO$_3$)$_4$ the copper ions have a very similar square-pyramidal geometry with one longer, apical bond to a nitrogen atom of the bpy or phen ligand, respectively. We therefore have speculated that (**L^1**)Cu and (2,9-dimethyl-1,10-*o*-phenanthroline)Cu^{2+} might have similar reactivities toward ApA. Although we could readily reproduce the results of Linkletter and Chin in aqueous solution, in an ethanol−water 19:1 medium at pH 6.6 neither (2,9-dimethyl-1,10-*o*-phenanthroline)Cu^{2+} nor (**L^1**)Cu (5 mM) cleave detectable amounts of ApA (0.1 mM) after 5 h according to reversed phase HPLC analysis.

Mechanism. A plausible reaction mechanism for the hydrolysis of BNPP by (**L^1**)Cu includes double electrostatic activation of the substrate by metal coordination and interaction with one of the ammonium groups, followed by nucleophilic attack of Cu coordinated hydroxide at phosphorus (Chart 1). Attack of coordinated hydroxide in a template reaction is a generally accepted mechanism of phosphodiester cleavage by metal complexes in which two *cis*-orientated coordination sites are available.[26,12a] Furthermore, it is known that ammonium groups facilitate phosphodiester cleavage by general acid catalysis.[27] Chart 1 combines these two features in the same molecule.

Although (**L^1**)Cu displays a square-pyramidal Cu geometry the metal-hydroxide mechanism in chart 1 is comparable to that proposed for square planar (bpy)Cu since an in-plane coordination of both phosphodiester and hydroxide to (**L^1**)Cu is obvious according to crystallographic and EPR spectroscopic results. The in-plane Cu−O bond lengths of **6** (1.96 ±0.04 Å) are very similar to the Cu−O distances of (bpy)Cu-complexes with in-plane N_2O_2-coordination.[20] In (trpy)Cu (trpy = 2,2′:6′,2′-terpyridine) only one in-plane coordination site is available for catalysis, and this complex is inactive for the hydrolysis of BNPP.[12a,28]

For (**L^1**)Cu and (**L^2**)Cu the Lewis acidity and the coordination geometry of the metal and the pK_a of coordinated water are expected to be quite similar. The large difference in reactivity is attributed to stabilization of the phosphorane intermediate by hydrogen bonding or proton transfer, respectively, in case of (**L^1**)Cu. Additionally, the X-ray crystal structure of **3** indicates that hydrogen bonding in the ground state might fix the substrate in a suitable orientation for the attack of copper coordinated hydroxide at phosphorus.

Molecular models based on the crystallographically determined square-pyramidal geometry of (**L^1**)Cu reveal no obvious possibility how both ammonium groups might simultaneously stabilize a coordinated phosphorane intermediate by hydrogen bonding. In our view participation of two $-NMe_2H^+$ groups is unlikely but cannot be completely ruled out.[29]

An alternative mechanistic pathway that includes nucleophilic attack of $-NMe_2$ at phosphorus should be discussed. Simple tertiary amines do not accelerate BNPP hydrolysis, but it was proposed that the diamines $Me_2N(CH_2)_nNMe_2$ ($n = 1-3$) catalyze BNPP cleavage by nucleophilic attack of $-NMe_2$.[27a] The pH-rate-profile of (**L^1**)Cu does not exclude this possibility. However, molecular models of a (**L^1**)Cu-BNPP complex show that formation of the pentavalent phosphorane by intramolecular nucleophilic attack of $-NMe_2$ at the phosphorus atom of coordinated BNPP is highly disfavored for steric reasons.

In summary, the ammonium-functionalized complex (**L^1**)Cu accelerates hydrolysis of BNPP 4×10^7-fold and is—to our knowledge—the most reactive complex of a divalent metal ion for the cleavage of this phosphodiester. (**L^1**)Cu is 1000 times more reactive than (**L^2**)Cu in which $-NMe_2H^+$ groups are replaced by $-NMe_3^+$ and 2900 times more reactive than (bpy)Cu. The crystal structure of [(**L^1**)$_2$Cu$_2$(1,3-μ-O$_3$POPh)$_2$(OH$_2$)$_2$]$^{4+}$ gives direct proof for intramolecular hydrogen bonding of one $-NMe_2H^+$ group with a coordinated phosphate ester. Based on pH rate profiles we propose that nucleophilic attack of Cu-OH to coordinated BNPP is operative in both (**L^1**)Cu and (**L^2**)Cu. The outstanding reactivity of (**L^1**)Cu is explained by hydrogen bonding of the coordinated phosphodiester with $-NMe_2H^+$ which provides an additional electrostatic activation and possibly fixes the substrate in close proximity to the nucleophile. (**L^1**)Cu may be regarded as a simple model for the highly efficient active site cooperativity of metal ions and NH-acidic amino acid side chains, a common functional motif of phosphoryl transfer enzymes.

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Supporting Information Available: X-ray structural information for **3** including positional and thermal parameters and bonding parameters (4 pages). See any current masthead page for ordering and Internet access instructions.

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[28] Interestingly, (trpy)Cu efficiently hydrolyses RNA and 2′,3′-cAMP. The reaction mechanism is essentially different from that of BNPP hydrolysis by (bpy)Cu.[12b,d] For a discussion, see: Bashkin, J. K.; Jenkins, L. A. *Comments Inorg. Chem.* **1994**, *16*, 77−93.

[29] To settle this question we have prepared [6-($Me_2HNCH_2C{\equiv}C$)-6′-($CH_3CH_2CH_2C{\equiv}C$)bpy](NO_3) which carries only one ammonium group. The copper(II) complex of this ligand appeared to be much more reactive than (**L^2**)Cu, but a detailed kinetic analysis was complicated by rapid Cu(II)−Cu(I) reduction. The reason for the reduction sensitivity of this complex is not clear.