# Hydrolysis of Double-Stranded DNA Promoted by Binuclear Cu(II) Complexes

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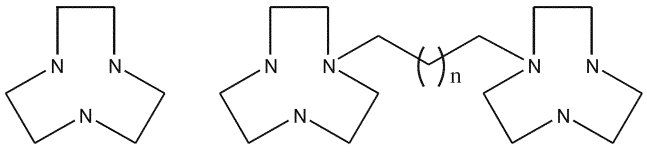
It is well known that natural metallohydrolases such as alkaline phosphatase and P1 nuclease contain more than two metal ions in the active site.[1] It has been reported that efficient catalysts for the hydrolysis of phosphate esters including DNA often carry binuclear Co(III), Cu(II), Zn(II), La(III) and Fe(III) ions, and yet mechanisms of DNA cleavage are not clearly established.[2]

Recently we have reported that the binuclear Cu(II)L2 complex (L2: 1,3-bis(1,4,7-triazacyclononyl)propane), cleaved DNA over 30 times faster than the corresponding mononuclear CuL1 complex (L1: [9]ane$N_3$) at pH 7.3 and 30 $^oC$.[3] According to the experimental results, the cleavage preceded most likely *via* hydrolytic pathway.[3,4] The observed rate enhancement in the CuL2 complex mediated DNA cleavage was attributed to cooperative role of the two Cu(II) ions. However it was surprising because the CuL2 complex showed the same reactivity as the CuL1 complex in hydrolyzing bis(4-nitrophenyl) phosphate (BNPP).

The different behavior towards BNPP and DNA hydrolysis by the CuL2 and the CuL1 complexes suggested that there must be other factors or mechanisms operating in the CuL2 complex mediated hydrolysis of DNA. The more negatively charged DNA than BNPP might facilitate the hydrolysis through the favorable binding to the catalyst, and the nature of the cooperativity between two Cu ions might be different in the hydrolyzing BNPP and DNA.

In this paper, we prepared the binuclear CuL3 complex (L3: 1,5-bis(1,4,7-triazacyclononyl)pentane), carrying the same positive charge as the CuL2 complex with cooperativity not being expected in hydrolyzing BNPP or 4-nitrophenylphosphate (NPP), and tried to evaluate contribution of the electrostatic interaction to the observed rate enhancement in the CuL2 complex mediated hydrolysis of DNA.




**L1**

n=1, **L2**
n=3, **L3**

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## Experimental Section

**Instruments**: Kinetic studies were carried out using Scinco-S3100 UV/Vis spectrophotometer. Gel imaging and analysis were performed using Kodak EDAS 290 gel documentation system.

**Materials**: The ligands, L2 and L3, were prepared by literature methods, including the orthoamide intermediate of L1.[5] Cu(II) complexes of L2 and L3 were prepared according to the known method by mixing ethanolic solution of the ligand and 2.0 equivalent of $Cu(NO_3)_2$, respectively.[6]

**Hydrolysis of BDNPP and NPP**: Cu complexes promoted hydrolysis of BDNPP and NPP was monitored by following the visible absorbance change at 400 nm (assigned to those of 2,4-dinitrophenolate and 4-nitrophenolate, respectively) at 30(±0.5) $^oC$. Addition of 0.10 M $NaNO_3$ had no appreciable effect on the rate of the hydrolysis. All the reactions were carried out either under pseudo-first order (BDNPP) or second-order (NPP) conditions. The rate constants were obtained by fitting the initial (<3%) concentration changes according to the first-order kinetics equation (correlation coefficient >0.98). The pH of the solution was maintained by 10 to 20 mM of corresponding buffers (MES: 6.5, MOPS: 7.3-8.0, CHES: >8.5). In a typical kinetic run, to a 2 mL of 0.25-0.5 mM of the catalyst, was added a 2.5-10 $\mu$L of 0.1 M stock solution of BDNPP in acetonitrile or NPP in water.

**Hydrolysis of ds-DNA**: Solutions (20-40 $\mu$L of total volume) containing 102 $\mu$M base-pair DNA (supercoiled pCMV-Myc from Clonetech. Labs) were mixed with varying concentrations of Cu complexes (5-100 $\mu$M) at ice bath. The pH of the solution was maintained with 10 mM HEPES (pH 7.3). About 4-5 $\mu$L of an aliquot was placed in an eppendorf tube and incubated at 28(±0.5) $^oC$. At certain time intervals, the reaction was stopped by adding gel loading buffer (bromophenol blue, xylene cyanol, 50% glycerol) containing 4 mM EDTA, and stored at -20 $^oC$ until analysis. Cleavage products were analyzed in 1% agarose gels. The gels were staining in TAE buffer containing 1 $\mu$g/mL ethidium bromide and DNA degradation was determined by Kodak 1D Image Analysis software. The intensity correction factor of 1.22 for supercoiled DNA (form I) obtained from the calibration experiments was utilized.[7] The decreases in form I DNA were plotted against time and they were fitted well with first-order exponential decay curve, in the case where enough amounts of the form I were remained during the reaction. All experiments were performed in triplicate.

The kinetic run by gel electrophoresis was reproducible within 20% error.

## Results and Discussion

Hydrolysis of NPP and BDNPP by the Cu complexes has been performed at 30 °C. The observed second-order rate constants are listed in Table 1. There were no differences in rate constants for BDNPP hydrolysis by three Cu(II) complexes. Under the experimental conditions used, no absorbance changes by the Cu(II) complexes were observed indicating dimerization of the complexes negligible.[8] In contrast to the hydrolysis of BDNPP, the CuL2 complex was two orders of magnitude faster in hydrolyzing NPP than the CuL1 complex. Binuclear Co(III) complexes by Czarnik and a binuclear Cu(II) complex by Chin showed ca. 10 and 500-fold rate enhancements in hydrolyzing NPP and ApA, respectively than the corresponding monomer did.[9] In both cases, rigid spacers such as phenyl and naphtyl groups were employed to bring about two metal ions in the close proximity and the double Lewis acid type of activation mechanism was suggested. It was interesting to observe the cooperativity between two Cu ions with flexible binucleating ligand such as L2. The CuL3 complex was 5 times more efficient than the CuL1 complex in hydrolyzing NPP, showing mild cooperativity.[10]

The hydrolysis of ds-DNA was followed by monitoring conversion of supercoiled plasmid DNA (form I), to the relaxed circular (form II) and the linear (form III) forms of DNA (Figure 1). Under the experimental conditions used, the form III formation by the CuL1 complex was negligible. The reaction was pH dependent, where the maximum cleavage was obtained around pH 7.5 to 8 (data not shown).[11] The second order rate constants for degradation of the form I to the form II by the CuL1, CuL2, and CuL3 complexes were $8.3 \times 10^{-1}$ $M^{-1}s^{-1}$, $2.8 \times 10^{-1}$ $M^{-1}s^{-1}$, 4.0 $M^{-1}s^{-1}$, respectively (Table 2). Plasmid DNA was cleaved at least 30 times faster by the CuL2 complex than the CuL1 complex. The CuL3 complex was about 5 times more active than the CuL1 complex.

**Table 1**. Observed second-order rate constants ($M^{-1}s^{-1}$)[a] for hydrolysis of phosphates mediated by Cu(II) complexes at pH 7.3 and 30 °C

| | CuL1 | CuL2 | CuL3 |
|---|---|---|---|
| NPP | $2.08 \times 10^{-4}$ | $1.19 \times 10^{-2}$ | $1.25 \times 10^{-3}$ |
| BDNPP | $1.25 \times 10^{-2}$ | $4.36 \times 10^{-2}$ | $2.08 \times 10^{-2}$ |

[a]pH 7.3 (20 mM HEPES), I = 0.1 M $NaNO_3$, [Cu complex] = 0.25-0.5 mM, [NPP] = 0.25-0.5 mM, and [BDNPP] = 2.5-5.0 $\times 10^{-5}$ M.

**Table 2**. The extent of DNA cleavage (%) at various concentrations of the Cu complexes at pH 7.3 and 28 °C

| Catalyst (μM) | Form I | Form II | Form III |
|---|---|---|---|
| CuL1 (40) | 59.3 | 40.7 | – |
| CuL2 (6) | 16.9 | 76.7 | 6.4 |
| CuL3 (10) | 56.3 | 43.6 | – |
| (20) | 48.1 | 51.9 | – |

[a]after 12 hours incubation: [DNA]=104 μM and 10 mM HEPES buffer used.

We previously proposed double Lewis acid type activation mechanism in the CuL2 complex mediated hydrolysis of NPP, which was known to be more effective for the hydrolysis of phosphate diesters of RNA type or phosphate monoesters.[2,3] In hydrolyzing BNPP and BDNPP, the CuL2 complex behaved like 2 equivalents of the CuL1 complex resulting almost the same reactivity as the CuL1 complex.[3] We did not expect the CuL2 complex to be efficient in cleaving phosphate diester backbone of DNA in this regard. We thought electrostatic interaction between the binuclear Cu complex and more negatively charged DNA over BNPP might be one of the factors participating in DNA hydrolysis. The CuL2 complex carries more positive charges than the CuL1 complex do. Ionic strength dependencies for nicking and linearization of plasmid DNA by the CuL2 complex shown in Figure 2, supported the strong electrostatic interaction.[12] The production of more negatively charged form III DNA by the CuL2 complex was inhibited at lower $NaNO_3$ concentration than the form II DNA production.

If the electrostatic interaction between DNA and the CuL2 complex contributes major part of the reactivity, the CuL3 complex should give the similar reactivity as the CuL2 complex in hydrolyzing DNA. The CuL3 complex carries the same positive charges as the CuL2 complex, with low possibility of cooperativity between Cu ions due to flexibility of the linker, pentyl group. In hydrolyzing DNA, the CuL3 complex showed the similar ionic strength dependence. It

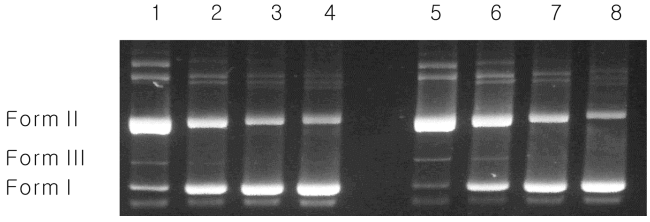


**Figure 1**. ds-DNA (pCMV-Myc: 102 μM bp) cleavage reaction by the Cu complexes at pH 7.3 (10 mM HEPES) and 28 °C; lane 1 and 5: DNA + CuL2(10 μM), lane 2 and 6: + CuL3(10 μM), lane 3 and 7: + CuL1(20 μM), lane 4 and 8: control (DNA only) after 4 and 8 hours, respectively.

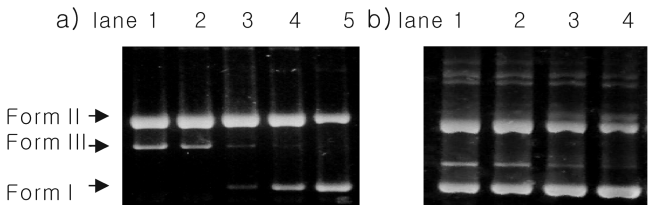


**Figure 2**. Ionic strength dependence of the Cu complexes mediated ds-DNA (pCMV-Myc: 102 μM bp) cleavage at pH 7.3 (10 mM HEPES) and 30 °C; (a) by the CuL2 complex: lane 1-5: 0, 10, 30, 50, and 100 mM $NaNO_3$, after 3 hours, (b) by the CuL3 complex: lane 1-4: 0, 10, 30, and 50 mM $NaNO_3$, after 6 hours.

was much less reactive than the CuL2 complex and slightly more reactive than the corresponding mononuclear the CuL1 complex in hydrolyzing DNA. Electrostatic interaction might facilitate the initial binding of the binuclear Cu complexes to DNA but it can not explain the observed rate enhancement in hydrolyzing DNA. It seems that the rate determining step involves the cleavage of phosphate diester bond of DNA, which depends largely on cooperative role of the Cu ions in the binuclear CuL2 complex. The nature of the cooperativity between two Cu(II) ions in the CuL2 complex, however, would be different from that found in hydrolyzing NPP, and coordination mode of the CuL2 complex to phosphate diester backbone of DNA are yet to be investigated. Since a mononuclear metal complex can act as a metal hydroxide, Lewis acid, and general acid or base, the role of the metal ions in the binuclear complex would be diverse and complicated.

In conclusion, the subtle structural changes in the ligands such as L2 and L3 result in large differences in the efficiency of the catalyst in hydrolyzing DNA. We are currently working on the hydrolysis of phosphate esters including DNA with rigid binuclear Cu complexes to find out the role of metal ions, based on the structures of the binuclear catalysts and the corresponding mechanisms.

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