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Phosphodiester modification by zinc metalated adenine polymer with carboxyl pendants

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Abstract—This report describes a novel carboxyl pendant containing adenylated polymeric template, its metalation with Zn (II), and manifestation of catalytic activity for the hydrolysis of model phosphodiester, bis(*p*-nitrophenyl) phosphate (bNPP), and plasmid cleavage. Observation of a bell-shaped pH-K_{obs} profile suggested influence of pH variation over hydrolysis rate. This metalated polymer also afforded facile relaxation of pBR322 supercoiled DNA, with an interesting reusability feature intricately associated with heterogeneous catalysis.

Redox inertness and hard–soft properties of zinc play an important role in its coordination to various donor environments in proteins thus making it available for biological activities.[1] Zinc coordinates to side chains of Cys, His, Asp, and Glu, with water playing a crucial role in catalytic zinc sites.[2] Several zinc enzymes and deoxyribozymes have been described for their nuclease activity pertaining to hydrolysis of DNA and RNA.[3] Interaction of zinc with nucleobases has been investigated[4] and there have been reports of structurally characterized zinc–adenine complexes.[5] Several examples of phosphate ester hydrolysis[6] and DNA cleavage by zinc complexes have also been reported.[7]

In the present paper, we report synthesis, characterization, and catalytic activity of **AP1-Zn** homopolymer. The effectiveness of **AP1-Zn** as a heterogeneous catalyst has been extensively studied by choosing routinely used phosphodiester substrate, bis(*p*-nitrophenyl)phosphate (bNPP). **AP1-Zn** assisted hydrolysis of bNPP monitored by time-dependent release of *p*-nitrophenolate anion ($\varepsilon_{400} = 1.65 \times 10^4\ M^{-1}\ cm^{-1}$). Attempts were also done to explore nuclease activity of the homopolymer toward super-coiled plasmid, pBR322 relaxation.

N-(6-purinyl)-caproic acid (**1**) was prepared according to a reported method and further derivatized to give a polymerizable monomer, 9-(4-vinylbenzyl)purine-6-yl amino caproic acid (**2**).[8] Site of alkylation was confirmed by spectroscopic evaluation and crystal structure studies (Figs. 1 and 2).[9] Acidic polymer (**AP1**) was synthesized using standard free-radical initiation chemistry.[10] **AP1** was metalated with zinc chloride to yield **AP1-Zn**.[8] (Scheme 1).

Compound **1** exhibited purine–purine dimerization with the help of carboxylate group hydrogen bonding with

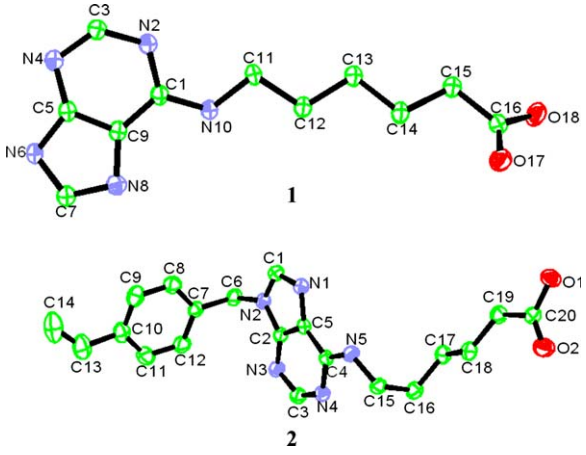

Figure 1. ORTEP diagram of **1** and **2**.

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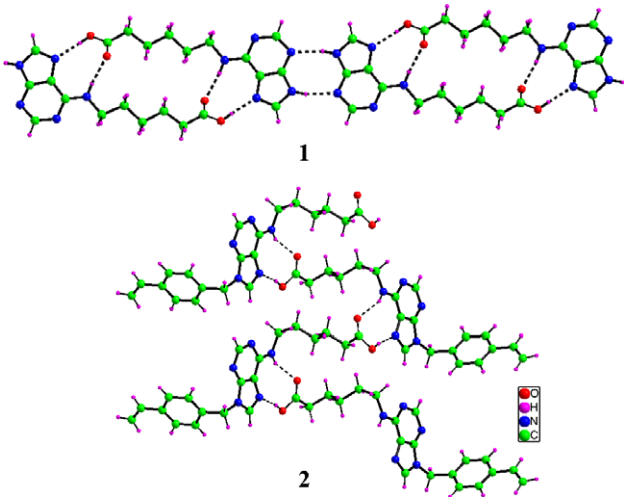
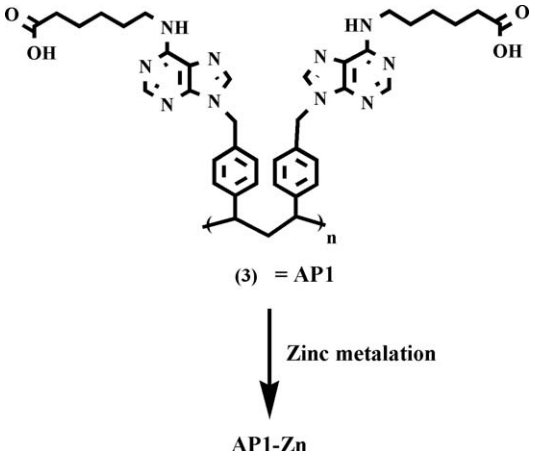


Figure 2. POVERAY diagram showing intermolecular hydrogen bonding in solid state structure of **1** and **2**. Color code: Green: Carbon; Blue: Nitrogen; Red: Oxygen; Pink: Hydrogen.



Scheme 1. Molecular structure of **AP1** polymer.

the Hoogsteen face of another modified purine, and this dimer afforded extended interaction in the solid state via base-pairing mediated by N3–H9 interaction. In case of **2**, alkylation at N9 resulted in the loss of dimeric structure, but a continuous hydrogen bonded supramolecular lattice was observed due to hydrogen bonding of carboxylate group with the Hoogsteen face.

Commonly employed activated phosphodiester substrate (bNPP) was chosen to evaluate hydrolytic potential of **AP1-Zn**.[11] Pseudo-first-order rate constants for bNPP hydrolysis catalyzed by **AP1-Zn** were determined for various pH values between 8 and 9.5. A bell-shaped curve with a maximum at pH 8.7 giving a clear indication that at this optimal pH, rate of bNPP hydrolysis was fastest (Fig. 3). The observed rate constant, k_{obs} for the release of *p*-nitrophenolate anion was $19.0 \times 10^{-5}\,min^{-1}$, which corresponded to $\sim 10^5$-fold enhancement over the uncatalyzed reaction.[12]

In comparison, two selected values for bNPP phosphodiester hydrolysis reported from the literature are:

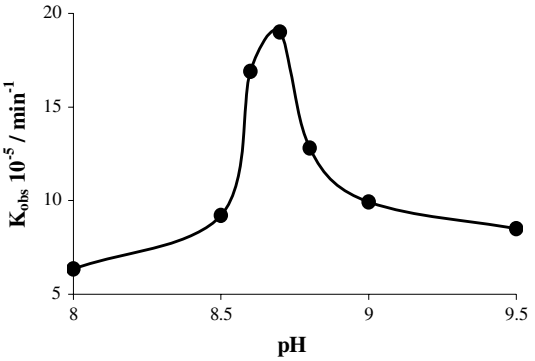


Figure 3. pH curve for bNPP hydrolysis catalyzed by **AP1-Zn**.

$9.1 \times 10^{-8}\,s^{-1}$ (pH 8.28, 50 °C) and $2.24 \times 10^{-6}\,s^{-1}$ (pH 9.2, 35 °C).[13]

Such profiles have been reported for other synthetic phosphodiesterases following hydrolytic pathway for phosphate ester cleavage.[14] It is established that transition metal ions lower pK_a of metal-bound water leading to the generation of hydoxyl ion, which can act either as a general base to activate solvent water or as a nucleophile to attack at the phosphorus center. We assume that our metal ion coordinated polymeric template activates water molecules and electrostatically mitigates the developing negative charge on the phosphate moiety to exhibit catalytic activity.

We also determined kinetic parameters for this catalyst under Michaelis–Menten conditions. Michaelis constant (K_m), maximal velocity (V_{max}), and turnover number (k_{cat}), from corresponding Lineweaver–Burk plot, were found to be 1.27 mM, 1.25×10^{-4} mM min^{-1}, and $4.8 \times 10^{-5}\,min^{-1}$, respectively (Fig. 4). Interestingly, the unmetalated homopolymers failed to assist phosphate ester hydrolysis in a control reaction confirming the importance of metal center in catalysis.

Coordinated zinc ions are responsible for the catalytic activity of **AP1-Zn**. We performed reaction arrest experiment to confirm the fact that adventitiously leached out metal ions are not responsible for hydrolytic assistance.

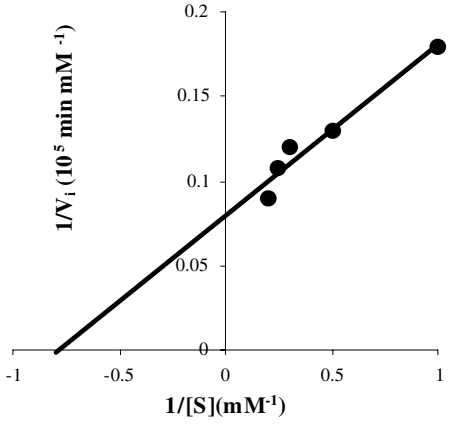


Figure 4. Lineweaver–Burk plot for bNPP hydrolysis catalyzed by **AP1-Zn**.

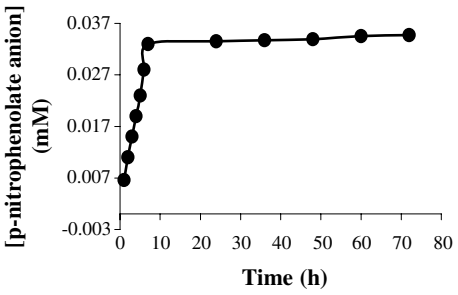


Figure 5. Reaction arrest experiment for bNPP hydrolysis catalyzed by **AP1-Zn** filtration.

In this experiment, **AP1-Zn** was filtered off from the reaction mixture after a reaction time of 7 h and hydrolysis of bNPP was further monitored up to 72 h at various time points. It was noticed that the hydrolysis reaction aborted soon after the removal of **AP1-Zn** and a lack of change in absorbance confirmed that hydrolysis occurred due to assistance rendered by **AP1-Zn** and not due to leached out zinc ions (Fig. 5).

AP1-Zn homopolymer was insoluble in aqueous and organic solvents and hence it could be easily recovered and recycled for further use. Recovered polymer was washed with aqueous methanol, acetone and air-dried prior to reuse. AAS analysis of the recycled **AP1-Zn** polymer confirmed that zinc has not leached out during the course of hydrolytic reaction (data not shown). Furthermore, kinetic data for fresh and reused polymer were comparable suggesting that **AP1-Zn** retains its catalytic potential through several cycles of hydrolytic reaction (supplementary data, Table 2).

When compared with the catalysts reported in the literature, **AP1-Zn** combines superior catalytic activity together with reusability. This property makes Zn-impregnated polymeric matrices more suitable as artificial oligozinc phosphate ester hydrolases compared to reported models.

AP1-Zn was further evaluated for hydrolytic cleavage of a natural substrate, supercoiled plasmid DNA pBR322. A time-course experiment was performed under heterogeneous conditions in cacodylate buffer containing pBR322.[15] Complete conversion of supercoiled form I to nicked form II was observed (Fig. 6, Lane 7), while unmetalated polymer **AP1** did not induce plasmid relaxation (Fig. 6, lane 2), confirming a crucial role of coordinated zinc ions in **AP1-Zn**. Interestingly, this cleavage reaction was completely inhibited in the presence of EDTA, which probably complexes zinc ions thus interfering with **AP1-Zn** catalytic activity (Fig. 6, lane 9). Similar effect of EDTA was observed for bNPP hydrolysis where its addition aborted hydrolytic activity presumably by zinc ion complexation (data not shown), thus verifying crucial role of zinc in hydrolysis of natural as well as non-natural substrates.

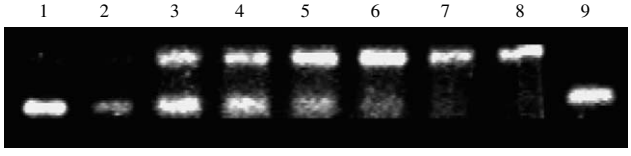


Figure 6. Nicking of pBR322 supercoiled DNA catalyzed by **AP1-Zn**. Each reaction contains 100 μg **AP1-Zn**. Lane 1: pBR322 alone; lane 2: pBR322 with unmetalated polymer, AP1 (60 h); lanes 3–8: pBR322 + **AP1-Zn** (12, 24, 36, 48, 60, 72 h, respectively); lane 9: pBR322 + **AP1-Zn** + EDTA (60 h).

Results presented above suggest that our water-stable metalated homopolymer, **AP1-Zn**, affords facile hydrolysis of phosphodiester hydrolysis substrates when compared to its unmetalated counterpart. Insolubility of **AP1-Zn** in reaction medium facilitates its separation subsequent to hydrolytic reaction. Recovered polymer retains its catalytic activity making recyclability of **AP1-Zn** as an extremely attractive feature for future investigations. Synthetic nuclease activity of **AP1-Zn** against pBR322 supercoiled DNA provides an indication that such reusable artificial models may become important tools for various biochemical applications.

Acknowledgment

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Supplementary data

Crystallographic data (excluding structure factors) for structures 1 and 2 have been deposited with the Cambridge Crystallographic Data Center. CCDC number for 1 is 247273; for 2 is 247274. Copies of this information can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK. [Fax: +44-1223/336-033; E-mail: deposit@ ccdc.cam.ac.uk]. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.07.077.

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8. Hausch, F.; Jäschke, A. *Tetrahedron* **2001**, *57*, 1261, 9-(4-vinylbenzyl)-purin-6-yl-amino caproic acid (2). *N*-(6-Purinyl)-caproic acid (1) (1.0 g, 4.0 mmol), 4-vinylbenzyl chloride (0.5 mL, 3.6 mmol), and sodium hydride (0.21 g, 8.8 mmol) were stirred in anhydrous DMF (20 mL) at 30 °C. After 12 h of stirring, solvent was removed under vacuum to dryness. Residue was dissolved in water, filtered, and pure product precipitated by addition of 1 N HCl, 0.95 g (2.5 mmol, 65%). R_f (silica gel) = 0.6 (CH_2Cl_2/MeOH/CF_3COOH 9:0.5:0.5). Mp 170 °C. ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 1.45–1.53(m, 2H); 1.69–1.79 (m, 4H); 2.35 (t, 2H, J = 7.3Hz); 3.6–3.63 (m, 2H); 5.24(d, 1H, J(H,H) = 11 Hz); 5.3 (s, 2H); 5.7 (d, 1H, J(H,H) = 18 Hz); 6.7 (dd, 1H, J(H,H) = 18 and 11 Hz); 7.2 (d, 2H, J(H,H) = 8 Hz); 7.36 (d, 2H, J(H,H) = 8 Hz); 7.7 (s, 1H); 8.4 (s, 1H). FABMS (M+1) = 366. *Zinc metalated AP1* (**AP1-Zn**). Potassium salt of AP1 (1.2 g) dissolved in MeOH (70 mL) and $ZnCl_2$ (0.75 g) was added to this solution. The reaction mixture was stirred for 12 h at room temperature. A white precipitate formed which was filtered, washed with methanol (4× 25 mL), acetone (2× 20 mL), and ether (2× 20 mL). Resulting metalated complex (1 g) was dried under vacuum. The metalated polymer, **AP1-Zn** was cream in color and insoluble in all common solvents and thus acted as heterogeneous catalyst in the hydrolytic reaction. The amount of Zn incorporated in the polymeric matrix was determined by AAS analysis and was found to be 16.7 mg/g of the polymer. The resulted zinc complex was used for phosphate ester hydrolysis and DNA cleavage reactions..
9. ^1H NMR spectra recorded using a JEOL 400 MHz spectrometer in $CDCl_3$ (Aldrich). FABMS recorded at RSIC, Lucknow, and AAS values recorded at FEAT Laboratory, IIT-Kanpur. *Single crystal X-ray studies*: Light green colored crystals of **1** were grown from methanol and acetone (1:1) solvent mixture by slow evaporation method and mounted on an Enraf Nonius FR590 CAD-4 diffractometer. Colorless prismatic crystals of **2**, suitable for X-ray studies, were grown from chloroform and acetone (2:1) mixed solvent by slow evaporation and mounted on diffractometer.
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11. *Phosphate ester hydrolysis*. All hydrolytic reactions were performed in duplicate in centrifuge tubes thermostated at 30 °C for bNPP hydrolysis. The assay mixture contained 3 mL of substrate solution of appropriate concentration prepared in 0.01 M *N*-ethylmorpholine (NEM) aqueous buffer in 90% aqueous methanol (pH 8.6). The reference cell contained substrate without metal conjugates, to correct for background hydrolysis. **AP1-Zn** weight was 1 mg mL^{-1}. For K_m and V_{max}, concentration of bNPP was 1.0–5.0 mM. For K_{obs}, concentration of bNPP was 12 mM. Control experiments of unmetalated AP1 alone, with phosphate ester substrates, did not accelerate hydrolysis, suggesting a magnificent purpose of coordinated Zn (II) ions in **AP1-Zn**. Initial velocities were determined as a function of time-dependent release of *p*-nitrophenolate anion at 400 nm ($\varepsilon_{400} = 1.65 \times 104\ M^{-1}\ cm^{-1}$), with a Shimadzu UV-160 spectrophotometer. Michaelis–Menten parameters were determined using corresponding Lineweaver–Burk plots. All hydrolytic reactions were performed at least over five half-lives of each substrate.
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15. *pBR322 cleavage experiment*. All cleavage reactions were performed by incubating 100 μg **AP1-Zn** (corresponding to 0.127 mM of zinc concentration, if the polymer was to be completely soluble in buffer) in sodium cacodylate buffer (10 mM, pH 7.5), at 35 °C, unless otherwise mentioned. Total reaction volume was 20 μL and weight of the DNA was 8 ng/μL of buffer. Two microliters of methanol was used for polymer wetting. All reactions were quenched with 5 μL of loading buffer containing 100 mM EDTA, 50% glycerol in Tris–HCl, pH 8.0, at regular time intervals. The samples were loaded onto 0.7% of agarose gel containing ethidium bromide (1 μg/mL) and were electrophoresed for 1 h at constant current, 70 mA. Finally gels were imaged on PC-interfaced Bio-Rad Gel Documentation system 2000.