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An Artificial Metalloribonuclease Based on Cu(II)-Terpyridine Complex Attached to Poly(ethylenimine)

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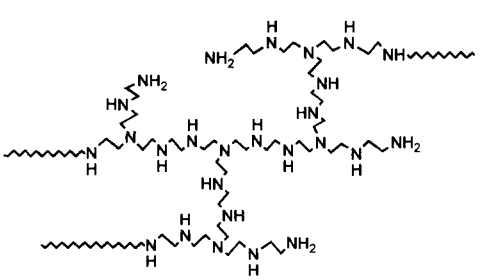
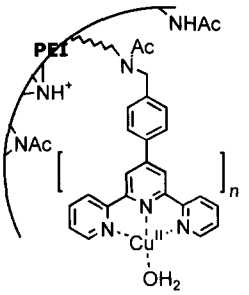
Abstract: Catalytic activity of Cu(II) ion complexed to terpyridine in hydrolysis of polyadenylic acid is considerably enhanced upon attachment to poly(ethylenimine) (PEI), especially at pH 6.5. This is attributed to cationic microenvironments created on PEI backbone and, possibly, to the general acid action of the ammonium ions of PEI.

There is much interest in the design of artificial ribonucleases in view of importance of sequence selective cleavage of RNA.[1] Metal ions are far more versatile than organic functional groups in catalyzing organic reactions and have been utilized as catalytic groups in many biomimetic catalysts.[2] Several metal complexes are known to catalyze hydrolysis of phosphodiester linkages. For example, effective catalysis by the Cu(II) complex (CuTP) of terpyridine in the hydrolysis of polyadenylic acid (poly(A)) has been reported.[3] Thus, CuTP is a good candidate for the catalytic group to be utilized in the design of artificial metalloribonuclease. One of the important subjects in design of artificial enzymes is improvement of activity of catalytic groups by interactions with the microenvironment and with functional groups provided by the backbone. In the present study, branchy poly(ethylenimine)[4] (PEI) is used as the backbone of the artificial metalloribonuclease to which CuTP is attached. In this paper, enhancement in the activity of CuTP through interaction with the PEI portion is reported.

PEI contains ethylamine as the repeating unit and, thus, is highly soluble in water. The molecular weight of the PEI used in the construction of artificial enzymes is ca. 60000, corresponding to 1400 monomer residues. Among the 1400 nitrogens present in PEI, ca. 25, ca. 50, and ca. 25 % are primary, secondary, and tertiary amines, respectively. The tertiary amines represent branching points on the polymer backbone, and PEI is highly branchy. The reaction of 4'-*p*-tolyl-2,2':6',2"-terpyridine with *N*-bromosuccinimide in CCl_4 in the presence of benzoylperoxide produced 4'-(*p*-bromomethylphenyl)-tolyl-2,2':6',2"-terpyridine (TP-Br). Through alkylation of amino groups of PEI with TP-Br, PEI was converted into TP-PEI, which was treated with acetic anhydride to acetylate primary and secondary amino groups of the PEI backbone leading to the formation of TP-AcPEI. PEI, TP-PEI, and TP-AcPEI were purified by repetitive dialysis before structural modification or kinetic measurements. By spectral titration with Cu(II) ion, it was estimated that each TP-PEI or TP-AcPEI molecule contains 27 TP residues on the average. The Cu(II) complex (CuTP-AcPEI) of TP-AcPEI was prepared by adding $CuCl_2$ (95 mol % of the TP residue) to TP-AcPEI. The average formation constant for each Cu(II) complex of TP unit in CuTP-AcPEI was estimated as $(1.46 \pm 0.10) \times 10^{14}$ at 25°C and pH 7.00 by a competition experiment[5] using EDTA as the competing ligand.



PEI



CuTP-AcPEI

Catalytic efficiency of CuTP has been previously measured in the hydrolysis of poly(A) containing 12-18 adenylate residues.[3] In the present study, poly(A) with the average degree of polymerization being ca. 300 was used as the substrate for CuTP-AcPEI and CuTP. Fragmentation of poly(A) by CuTP-AcPEI and CuTP is evidenced by electrophoresis (e.g., Fig. 1). Fig. 1 shows that poly(A) and its cleavage products are mixtures of polymers with various sizes. Fig. 1 further reveals that poly(A) is much more rapidly cleaved into small pieces by CuTP-AcPEI than by CuTP at pH 6.50. AcPEI, the acetylated PEI derivative without the CuTP groups, did not promote degradation of Poly(A), revealing that CuTP is essential for the action of CuTP-AcPEI.

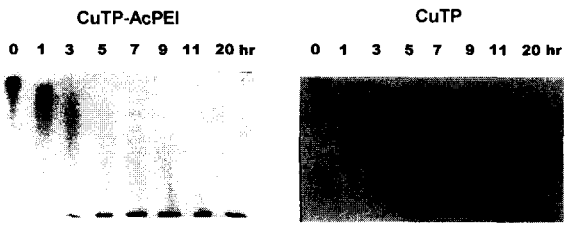


Fig. 1. Separation of cleavage products by electrophoresis (15 % polyacrylamide-7 M urea gel)[6] after incubation of poly(A) (8.22×10^{-5} res M) with CuTP-AcPEI or with CuTP at pH 6.50 (0.05 M Mes) and 50°C. Concentration of the CuTP moieties was 9.03×10^{-6} M.

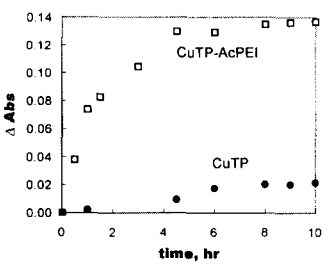


Fig. 2. Abs change at 276 nm observed during incubation of poly(A) with CuTP-AcPEI or CuTP under conditions of Fig. 1.

Since the substrate is a mixture of polymers, kinetic measurement by following the decrease in the density of the electrophoretic band for the parent poly(A) was not feasible. Instead, relative activity of CuTP-AcPEI and CuTP at pH 6.5-8 was quantitated spectrophotometrically. The progress in the fragmentation of poly(A) is accompanied by changes in UV spectrum. In Fig. 2 is illustrated the absorbance (Abs) change observed at 276 nm during incubation of poly(A). UV spectra of the product solutions obtained by incubation of poly(A) with CuTP-AcPEI and with CuTP were identical. The Abs change observed during the incubation of poly(A) with CuTP-AcPEI or CuTP is not proportional to the number of phosphodiester linkages cleaved.[7] Nevertheless, Abs changes accompanying cleavage of poly(A) may be used as a measure of relative reactivity.

The apparent initial velocity (v_o) was defined as $[\Delta Abs/\Delta t]_{t=0}[\Delta S/\Delta Abs]$ and was calculated by analyzing Abs increase according to the statistical method reported in the literature.[8] Conversion factor $\Delta S/\Delta Abs$ was arbitrarily taken as the initial residue molar concentration (S_o) of poly(A) divided by total Abs change observed during the reaction. Studies with CuTP-AcPEI were not extended to pH 6 and lower pHs due to precipitation apparently caused by extensive complexation between CuTP-AcPEI and poly(A). For Michaelis-Menten kinetics, initial velocity is proportional to S_o when $K_m >> S_o$. For the data illustrated in Fig. 3, v_o is proportional to S_o with the slopes representing v_o/S_o.

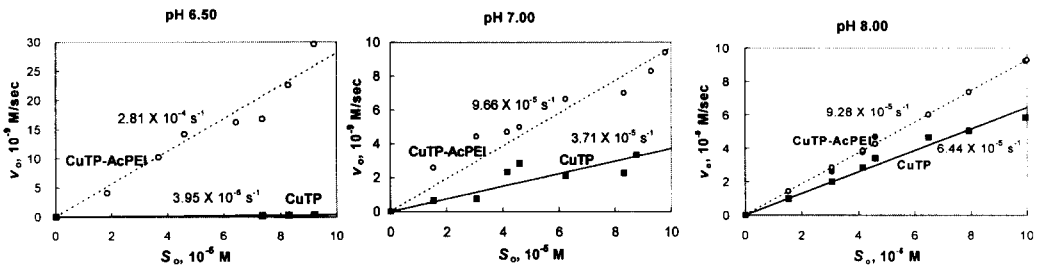
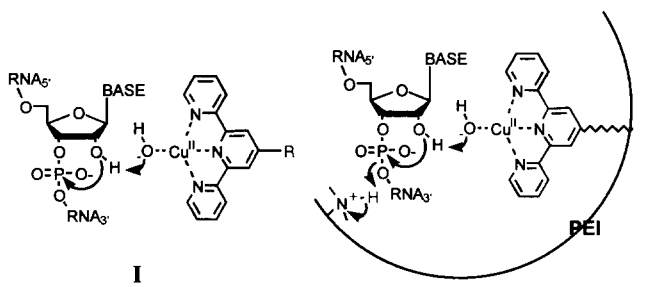


Fig. 3. Plot of v_o vs. S_o for hydrolysis of poly(A) catalyzed by CuTP-AcPEI or CuTP at 50°C and pH 6.50, 7.00, or 8.00. Catalyst concentration was 9.03 X 10^{-6} M in terms of the concentration of the Cu(II) center. The numbers are slopes of the straight lines.

Since CuTP is known to hydrolyze the phosphodiester linkages of RNA,[3] fragmentation of poly(A) by CuTP-AcPEI is likely due to hydrolysis of phosphodiesters. The mechanism of catalysis by CuTP in hydrolysis of RNA was proposed as I, in which the hydroxide ion coordinated to the Cu(II) ion acts as a general base to assist the nucleophilic attack by the ribosyl hydroxyl group.[3] The pK_a of the water molecule bound to the Cu(II) ion of CuTP is 8.2.[3] Thus, CuTP loses its catalytic activity at lower pH values due to protonation of the hydroxoCu(II) ion. The pH dependence of v_o/S_o for the CuTP-catalyzed hydrolysis of poly(A) agrees with this mechanism.



I II

The results of Fig. 3 and electrophoresis (not shown for pH 7.00 and 8.00) indicate that CuTP-AcPEI is markedly more effective than CuTP at pH 6.50 whereas they possess similar activity at pH 8.00. For CuTP-AcPEI, the activity is higher at pH 6.50 than at pH 7.00 or 8.00. The polycationic microenvironments provided by the protonated tertiary amino groups of the PEI backbone would promote ionization of the

Cu(II)-bound water molecule, lowering the optimum pH for the catalysis. This alone, however, does not account for the higher catalytic activity of CuTP-AcPEI at pH 6.50 compared with pH 7.00 or 8.00. Another catalytic factor, which operates at pH 6.50 but becomes less important at higher pHs, is needed to explain the high catalytic activity of CuTP-AcPEI at pH 6.50. Protonation of the tertiary amino groups would facilitate complexation between CuTP-AcPEI, a polycation, and poly(A), a polyanion, as evidenced by precipitation at pH $\leq$ 6. Complexation of poly(A) to CuTP-AcPEI would accelerate the reaction between the two polymers. Another possibility that cannot be ruled out at present is the general acid catalysis by the tertiary ammonium ion as illustrated in II. Due to hydrophobic microenvironments and unfavorable electrostatic interactions among ammonium ions, amino groups of PEI derivatives are mostly deprotonated at pH >7.[9] The diminishing effect of the ammonium ions of CuTP-AcPEI at pH 7-8 indicates that they are largely deprotonated at pH >7.

Results of the present study demonstrate that the catalytic activity of CuTP in hydrolysis of poly(A) is considerably improved by attachment to PEI through interaction with the tertiary amino groups of PEI backbone. Although it is not possible at present to decide whether the catalytic effect of the protonated tertiary amino groups originates from their general acid activity or from the polycationic microenvironment, it is clear that more effective artificial metalloribonucleases could be obtained by construction of active sites comprising CuTP and other catalytic elements on PEI.

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