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Note

Effective heterogeneous hydrolysis of phosphodiester by pyridine-containing metallopolymers

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Abstract

The copper (II) complex of a simple pyridine- and amide-containing copolymer serves as an effective catalyst for heterogeneous hydrolysis of the prototypical phosphodiester substrate bis(*p*-nitrophenyl)phosphate at pH 8.0 and 25 °C. The catalysis has a first-order rate constant of $k_{\mathrm{cat}} = 8.3 \times 10^{-6}\ \mathrm{s}^{-1}$, corresponding to a catalytic proficiency of 75-thousand folds relative to the uncatalyzed hydrolysis with a rate constant of $k_0 = 1.1 \times 10^{-10}\ \mathrm{s}^{-1}$ in aqueous buffer solution at pH 8.0. This observation suggests that polymers can be designed to include various functional groups feasible for effective metal-centered catalysis of phosphodiester hydrolysis.

Keywords: Phosphoester; Hydrolysis; Metal; Cu; Metallopolymer; Polymer

1. Introduction

A number of functional and reactive polymers have recently been developed to show broad applications in catalysis, such as hydrolytic and oxidative catalysis [1]. The latter includes oxidative cleavage of nucleic acids [2], and the former is involved in many important biological, industrial, and environmental processes. For example, the reversible hydrolysis of phosphoester bonds and phosphorylation are the key steps in biological signaling, which trigger many normal and disease-related metabolic pathways [3]. Hydrolysis also plays a critical role in degradation of nucleic acids, phosphoester-containing pesticides, and nerve agents, including the G-agents (Sarin and Soman) and the VX-agent [4]. During hydrolysis, the nucleophilicity of a water molecule is greatly enhanced by a general base or a metal center and becomes substantially more acidic compared to free water molecules [5,6], which renders hydrolysis possible around neutral pH. The substrate can be significantly polarized by the metal center and becomes more susceptible to nucleophilic attack [5]. A number of metal complexes, including those of Co^{3+}, Cu^{2+}, Zn^{2+}, and lanthanides (III), have been synthesized in recent years as models for different families of metallohydrolases, such as peptidases, nucleases, and phosphoesterases [6]. The investigation of these model complexes has provided further insight into the mechanism of metal-centered hydrolysis.

Heterogeneous catalysis has been well recognized to be a convenient process in industry and laboratory owing to easy separation of the products, recovery of the catalysts, and incorporation into a continuous flow process. However, it has not been widely applied to hydrolytic processes. Some recent reports about effective hydrolysis by metal centers incorporated onto silica- and organopolymer-based matrices suggest potential applications of heterogeneous hydrolysis [7]. In this

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communication, we demonstrate that an easily prepared metallopolymer system can perform proficient heterogeneous hydrolysis toward phosphodiester bond, which can thus serve as a functional model system to provide further insight into metal-centered hydrolysis of phosphoesters.

2. Experimental

The reagents 2- and 4-vinylpyridine were obtained from Across Organics (Fair Lawn, NJ), acrylamide from BioRad (Richmond, CA), and the buffers HEPES, CAPS and MOPS, sodium acetate, Chelex resin, and bis(*p*-nitrophenyl) phosphate (BNPP) from Sigma-Aldrich (St Louis, MO). The buffer solutions were treated with Chelex resin to remove any trace amount of metal ions. Copolymers of 4-vinylpyridine (4Vp) and acrylamide (Ac) were prepared according to published procedures [8] by mixing different ratios of freshly distilled 4VP and recrystallized Ac and the initiator 2,2′-azoisobutyronitrile (AIBN) in DMF to produce copolymers of different average repeating units (RU) of $4Vp_x/Ac_y$. The stoichiometry of the RU of the copolymer was found to vary with the 4Vp/Ac ratio and the amount of AIBN in the reaction mixture. The copolymers were precipitated in ethyl acetate, then filtered and dried in a vacuum oven at 40 °C. Copolymers of 2-vinylpyridine (2Vp) and Ac were prepared with the same method as the 4Vp–Ac copolymers. The formation of the polymers was verified with a Bruker ADX250 NMR spectrometer. A total recycle time of ~10 s was used for spectrum acquisition to ensure complete relaxation of the ^1H NMR signals.

The hydrolytic activity of the metallopolymer complex was determined by measuring the initial rate of the hydrolysis of BNPP in 1:1 methanol/HEPES buffer of 25 mM at pH 8.0 and 25 °C, wherein the increase of the absorption at 405 nm ($\epsilon = 17500\ M^{-1}\ cm^{-1}$ due to the hydrolytic product *p*-nitrophenolate) with time was obtained on a Varian Cary 3E spectrophotometer. A plot of the formation of the product with respect to time gives the rate.

Upon binding with Cu^{2+}, the resulting complex precipitates out as blue solid, which is the active catalyst toward phosphodiester hydrolysis. The optimum metal-to-RU stoichiometry for the catalysis was determined by titrating 1.0-mM polymer solution with Cu^{2+} and checking the resulting heterogeneous solution for activity toward hydrolysis of 1.0-mM BNPP, a prototypical phosphodiester substrate, at pH 8.0 and 25 °C.

The stoichiometry of metal complexes can also be conveniently determined by means of the Job plot [9], wherein the optical density is monitored under different mole fractions of the metal (X_M) or the ligand ($X_L = 1 - X_M$) with a constant total concentration of the metal and the ligand ([M] + [L]). The mole fractions that exhibit the maximum absorption afford the stoichiometry of the complex. Thus, a maximum at $X_M = X_L = 0.5$ reflects the formation of a complex of X_L and a maximum at $X_M = 0.33$ ($M_L = 0.67$) indicates the formation of a complex of ML_2.

3. Results and discussion

Vinyl pyridine and acrylamide were chosen for the preparation of copolymers that are expected to bind transition metals through the pyridine moiety and form H-bonding using the amide, thus may loosely mimic the active-site environment of various metallo-hydrolytic enzymes [5]. The formation of the polymers can be verified with ^1H NMR spectra, which exhibit very broad signals of up to ~500 Hz for the polymers and the disappearance of the sharp monomer signals. The broadness of the signals is the consequence of the very slow rotational correlation time of the "polymer molecules" and the irregular monomer sequence in the polymers.

Depending on the Vp/Ac ratio and the amount of the initiator in the preparation, copolymers with different amounts of Vp and Ac can be obtained. The use of a molar ratio of 4Vp:Ac = 1:3 in the presence of 1% molar amount of AIBN produced a copolymer with a stoichiometry of $4Vp_3Ac_1$ for the RU based on the integration of the ^1H NMR signals of the pyridine ring and the amide NH_2 protons (Fig. 1). The latter signals disappear upon addition of a few drops of D_2O into the polymer in DMSO. The chemical shifts of the solvent exchangeable NH_2 signals in the copolymer are consistent with those of Ac at 6.4 and 6.9 ppm. The integration gives a 4Vp:Ac ratio of 3:1, with $4Vp_3Ac_1$ as the average RU for this copolymer. The stoichiometry of $4Vp_3$-Ac_1 gives an apparent RU formula mass of 386.5 Da, which was used for the calculation of the mole fractions. The

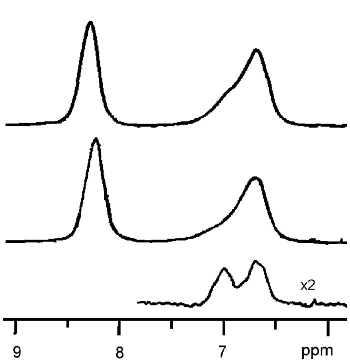


Fig. 1. The ^1H NMR signals of the pyridine ring and the amide NH_2 protons of the copolymer in DMSO (top trace) and in DMSO upon addition of a few drops of D_2O, where the NH_2 signals are wiped out (middle trace). The change can be clearly seen in the difference spectrum which reveals the NH_2 signals (bottom trace).

molecular mass of the copolymer prepared herein was estimated to be greater than 30 kDa based on ultrafiltration with a membrane of a molecular-weight-cut-off of 30 kDa.

This copolymer family has been previously determined to bind various metal ions [8]. We observed a linear increase in hydrolytic activity (discussed later) upon Cu^{2+} addition until one equivalent per RU was added (Fig. 2), indicating a 1:1 stoichiometry of metal binding to each RU necessary for optimum catalysis. To ensure that the activity is due to the insoluble metallopolymer complex, the heterogeneous Cu^{2+}–$4p_3Ac_1$/solvent mixture was centrifuged and the solid and the supernatant were evaluated for activity towards hydrolysis of 1.0-mM BNPP. The activity of the supernatant was negligible, while the activity of the solid matched that observed for the initial heterogeneous solution, indicating a heterogeneous catalysis.

To further confirm the metal-to-polymer stoichiometry of the metallo-polymer that exhibits the hydrolytic activity, a Job plot was constructed. Since the Cu^{2+} complex of the copolymer is not soluble, it is necessary to modify the conventional optical Job plot [9] into an "activity Job plot" in which the activity toward the hydrolysis of BNPP instead of the optical density of the complex was determined with respect to the mole fraction of Cu^{2+} (X_{Cu}) or polymer RU ($X_{RU} = 1 - X_{Cu}$) at a constant ($[Cu^{2+}] + [RU]$) of 2.0 mM. The maximum in the activity Job plot is found at $X_{Cu} \sim X_{RU} \sim 0.5$, i.e., at $[Cu^{2+}] = [RU] \sim 1.0$ mM (Fig. 2, inset), indicating that the predominant active species is the metallopolymer with a stoichiometry of Cu^{2+}:RU = 1:1 which is consistent with the stoichiometry obtained from the titration of Cu^{2+} to the polymer at a constant [RU] (Fig. 2).

The plot of the initial rate for the hydrolysis of 1.0 mM BNPP by various amounts of the complex in terms of $[Cu^{2+}$–RU] is linear (data points not shown), which affords an observed rate constant k_{obs} of 1.9×10^{-6} s^{-1} for the rate law, rate = $k_{obs}[Cu^{2+}$–RU]. The polymer and the metal ion separately did not show noticeable activity under the same conditions and time frame. Although BNPP has been generally acknowledged to be a substrate quite accessible to hydrolysis due to the very good leaving group *p*-nitrophenol, its auto hydrolytic rate is still *extremely slow* with a rate constant k_0 of 1.1×10^{-11} s^{-1} at pH 7.0 and 25°C, [10] i.e., 1.1×10^{-10} s^{-1} at pH 8.0, considering OH^- as the nucleophile, or a similar value of 3.76×10^{-10} s^{-1} under the experimental conditions of a mixed solvent. The hydrolysis of BNPP by the Cu^{2+}-copolymer complex thus shows a significant observed rate enhancement (k_{obs}/k_0) of 4800–18 000 folds with respect to the auto-hydrolysis of BNPP.

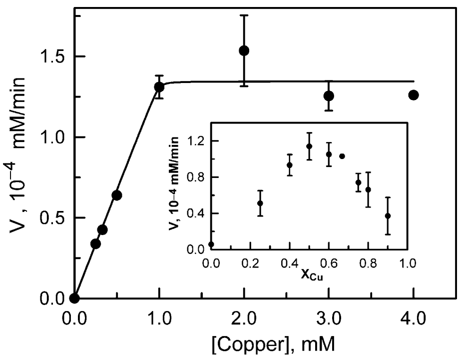


Fig. 2. Titration of Cu^{2+} into 1.0 mM RU of $4Vp_3Ac_1$ copolymer, monitored with the activity toward the hydrolysis of 1.0 mM BNPP in 50% methanol solution of 25 mM HEPES at pH 8.0 and 25 ° C. Inset shows the "activity Job plot" of Cu^{2+}–$4Vp_3Ac_1$ in which the initial rate of BNPP hydrolysis (1.0 mM) is plotted against X_{Cu} at a constant $[RU] + [Cu^{2+}]$ of 2.0 mM.

In order to establish the rate law for the heterogeneous BNPP hydrolysis by this Cu^{2+}-polymer complex, the rate is further determined at various BNPP concentrations at 25 °C. To ensure efficient complexation of the metal under all the experimental conditions during the kinetic studies (particularly at high pHs wherein metal ions can easily precipitate out as hydroxides), an excess amount of the polymer ($RU/Cu^{2+} \sim 2.5$ in terms of equivalent) is added to the reacting solution. The rate with respect to [BNPP] is found to be hyperbolic (Fig. 3), which indicates that the hydrolysis does not follow simple second-order kinetics, but probably a pre-equilibrium kinetics similar to surface catalysis. This kinetics can be described as the binding of the substrate BNPP (S) to the metal center to form an intermediate polymer–Cu(II)–S complex, followed by conversion of the bound substrate to the products (Eq. 1). The rate law for this reaction can be obtained with steady-state approximation and an assumption that the amount of the bound S is much less than that of the free S in solution, which is expressed as Eq. (2), in which $K' = (k_1 + k_{cat})/k_1$ is the virtual dissociation of the bound S. The data can be well fitted to Eq. (2) to give a first-order rate constant $k_{cat} = 8.3 \times 10^{-6}$ s^{-1} (under

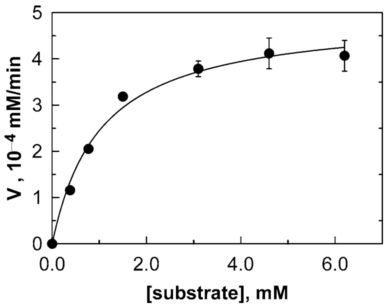


Fig. 3. Plot of the initial hydrolytic rate of BNPP by 1.0 mM $[Cu^{2+}$–RU] of the $4Vp_3Ac_1$ copolymer at pH 8.0 and 25 °C. The solid curve is the best fit to a pre-equilibrium rate law (Eq. (2)).

pseudo-first order conditions of high $[S] \gg K'$), $K' = 1.0$ mM, and a second-order rate constant $k_{\text{cat}}/K' = 8.3 \times 10^{-3}$ $\text{M}^{-1}\,\text{s}^{-1}$ at low $[S] \ll K'$ (Fig. 3). This good fitting verifies the pre-equilibrium pathway for the catalysis shown in Eq. (1), indicating direct substrate binding with the metallopolymer.

$$\text{Polymer-Cu(II)} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Polymer-Cu(II)–S} \xrightarrow{k_{\text{cat}}} \text{Polymer-Cu(II)} + \text{Prod}, \tag{1}$$

$$\text{rate} = \frac{k_{\text{cat}}[\text{Polymer-Cu}][\text{S}]}{K' + [\text{S}]}. \tag{2}$$

The hydrolysis of BNPP by the Cu^{2+}-polymer affords a significant catalytic proficiency [11a] k_{cat}/k_0 of 7.5×10^4 (or 2.0×10^4) folds in terms of the first-order rate constant with respect to the auto-hydrolytic rate constant of the substrate in buffer at pH 8.0 (or in a mixed solvent system under the experimental conditions herein), which represents a decrease of the half-life ($\ln 2/k$) of BNPP from 200 years to less than a day, and an enormous second-order catalytic proficiency [11b] $(k_{\text{cat}}/K_{\text{m}})/(k_0/55.5)$ of 4.1×10^9 folds. The latter is quite significant, since a large rate enhancement still exists even at very low substrate concentrations of <1.0 mM. The rate constants observed here are comparable or higher than what have recently been reported for a heterogeneous Cu^{2+} complex of an adenine polymer at pH 8.0 ($k_{\text{cat}} = 1.13 \times 10^{-6}$ s^{-1}, $k_{\text{cat}}/K' = 3.04 \times 10^{-5}$ $\text{M}^{-1}\,\text{s}^{-1}$) [7d], Cu^{2+}-triazacyclononane immobilized onto silica surface at pH 7.8 [7b] (a first-order rate constant of about 6×10^{-7} s^{-1} estimated from the initial rate of the maximum catalysis, i.e., cycle 7 in Fig. 2 of the reference) or immobilized on a polymer (0.5–5.2×10^{-6} s^{-1}) [7g], Cu^{2+}-bipyridine linked polymer (2.3×10^{-5} s^{-1}) [7f], a poly(ethylene glycol)-supported Cu^{2+}-triazacyclononane complex at pH 7.8 (6.0–9.6×10^{-6} s^{-1} and 4.3–5.0×10^{-3} $\text{M}^{-1}\,\text{s}^{-1}$) [12], and the homogenous Cu^{2+}-triazocyclononane complex at pH 7.0 (5.0×10^{-7} s^{-1}) [13].

The pH is one significant factor in hydrolytic reactions. Base hydrolysis becomes the predominant hydrolytic pathway at high pH, where OH^- serves as the nucleophile. Consequently, hydrolysis may not take place easily under neutral conditions owing to the low concentration of OH^- and the much weaker nucleophilicity of water molecule. The presence of a metal center in the active site of metallo-hydrolytic enzymes [5] can greatly increase the local concentration of OH^- in the active site in the form of metal-coordinated OH^- due to the high Lewis acidity of the metal center. The effect of pH on the hydrolysis of 1.0-mM BNPP by the Cu^{2+}-polymer complex was investigated in the pH range of 4.5–10.0 to reveal the nucleophile (Fig. 4). The data can be fitted to a single-ionization process to reveal an ionization constant of 6.70 ± 0.07 important for the catalysis. This ionization is attributable to a nucleophilic water coordinated to the Cu^{2+} in the complex because no other groups are expected to ionize around neutral pH. This value indicates a dramatic enhancement of the deprotonation of water with a $\text{p}K_{\text{a}}$ of 15.74 by about 10^9 folds (i.e., $10^{[-6.70-(-15.74)]}$) by this Cu^{2+}-copolymer complex. The low $\text{p}K_a$ value of the nucleophilic water is consistent with the high Lewis acidity of the Cu^{2+} center. This result confirms a metal-centered mechanism for the hydrolysis of BNPP by a coordinated hydroxide, but not by free OH^-. The high hydrolytic rate may also partially be attributed to a high local $[\text{OH}^-]$ at the metalloactive center.

On the basis of the results discussed above, an interaction scheme between the substrate and the Cu^{2+} active site can be proposed as below. The formation of Cu^{2+}–$4\text{Vp}_3\text{-Ac}_1$ centers is revealed from stoichiometric metal titration monitored with hydrolytic activity. The binding of the substrate to the metal active-center is demonstrated by the pre-equilibrium kinetics. This binding further polarizes the PO bonds for easier nucleophilic attack. An interaction of the substrate with a proximal amide in the polymer chain may be possible, which polarizes the PO bond even further and may also stabilize the charge on the substrate. The Lewis basicity and nucleophilicity of a coordinated water is significantly enhanced by the metal center and possibly by H-bonding with an amide group on the polymer chain. The significant enhancement in the rate by 7.5×10^4 folds reflects a significant decrease of 27.8 kJ/mol in activation free energy, which can be achieved by the involvement of one to two H-bonding interactions at the transition state. The hypothesis that the amide group in this metallopolymer might serve as a general base and/or as a H-bond donor/acceptor during the catalysis to engender the high hydrolytic activity will be verified in future

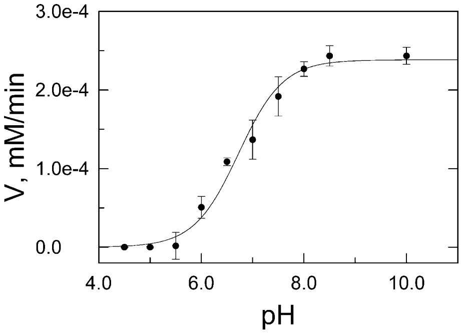
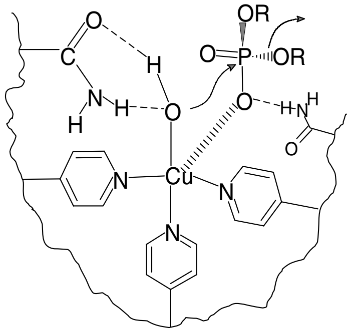


Fig. 4. The pH dependence of the initial rate for the hydrolysis of 1.0-mM BNPP by Cu^{2+}-polymer complex (1.0 mM $[\text{Cu}^{2+}]$) in the presence of excess amount of the polymer to prevent metal hydroxide precipitation at high pHs. The data are fitted to a single-ionization process according to the equation $V = V_{\text{limt}}/1 + ([\text{H}^+]/K_{\text{a}})$ in which V_{limt} is the rate for the deprotonated active species and K_{a} the ionization constant.

studies of metallopolymers possessing different functional groups. [1]



To provide further information about the design of catalytic metallopolymers, copolymers of 2VP and Ac (2VpAc) were also prepared with the same procedures for the preparation of 4VpAc. It is noteworthy that a Cu^{2+}–2VpAc complex shows 100 times lower activity compared to the Cu^{2+}–4VpAc complex under the same conditions. Currently, work is under way to further investigate the specific factors that are responsible for the significant activity of the Cu^{2+}–4VpAc metallopolymer toward phosphodiester hydrolysis.

To demonstrate that the Cu^{2+}–4VpAc complex is not only efficient but also reusable due to its heterogeneous nature, the complex was recovered by centrifugation, washed repeatedly with buffer and methanol, and added to freshly prepared solutions of BNPP at 1.0–4.0 mM. It was found that the recycled Cu^{2+}–4VpAc was able to continuously catalyze the hydrolysis of BNPP concentrations even up to eight times with considerable activities. Owing to the powdery texture of the metallopolymer, it can be recovered to ~80% of the original mass after four cycles which accounts for the loss of ~20% activity. Future design of the polymer texture, such as cross linkage and/or beaded and porous structure, is expected to enhance the reusability of the metallopolymer.

4. Concluding remarks

Cu^{2+} is unique among first-row transition metal ions in hydrolytic chemistry, wherein it can activate a few hydrolases (including serralysin [14] and astacin [15]) to a great extent while show negligible activation toward some other hydrolases such as carboxypeptidase A and carbonic anhydrase [16]. To gain further understanding of metal-centered hydrolysis and design more effective hydrolytic catalysts, it is essential to solve the puzzle about why and why not Cu^{2+} can activate these enzyme systems for hydrolysis. We present in this communication the investigation of the Cu^{2+} complex of a simple pyridine- and amide-containing copolymer family that exhibits a significant activity toward phosphodiester hydrolysis, which provides another model system for further investigation of Cu^{2+} -centered hydrolytic chemistry. The simplicity in the preparation of this 4VpAc copolymer and the high hydrolytic activity of its Cu^{2+} complex suggest potential application of this family of metallopolymers in hydrolytic chemistry. Moreover, since Cu^{2+} is a redox-active ion, Cu^{2+}-polymer systems may also serve as oxidative catalysts for further investigation of Cu-centered oxidation and oxygenation chemistry. [2]

Acknowledgements

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