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Remarkable reactivity of alkoxide/acetato-bridged binuclear copper(II) complex as artificial carboxylesterase

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Abstract Bromo-containing binuclear Schiff base copper(II) complex, Cu₂L(OAc), with an alkoxo/acetatobridged moiety was employed as a model of carboxylesterases to promote the hydrolytic cleavage of *p*-nitrophenyl picolinate (PNPP). Furthermore, the reactivity of a mononuclear complex (CuHL) was evaluated for comparing it with that of binuclear one. The results reveal that the asprepared binuclear Cu₂L(OAc) efficiently accelerated the hydrolysis of PNPP, giving rise to excess four orders of magnitude rate enhancement in contrast to the un-catalyzed reaction. Cu₂L(OAc) represented an enzyme-like bellshaped pH-responsive kinetic behavior. Moreover, the binuclear one is more reactive than its mononuclear analogue (CuHL) by two orders of magnitude. The total efficiency of Cu₂L(OAc) is about 61-fold than that of its mononuclear analogue, CuHL. In addition, a contrast experiment reveals that binuclear Cu₂L(OAc) displayed good activity in the hydrolysis of PNPP as well another active ester, i.e., S-2-benzothiazolyl 2-amino-alpha-(methoxyimino)-4-thiazolethiolacetate (AE-active ester). Noteworthyly, it was found that mononuclear one inspired more obvious rate enhancement in the hydrolysis of AE-active ester relative

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to PNPP hydrolysis. The estimated pK_{a1} of bound water on the binuclear Cu₂L(OAc) using second derivative method (SDM) is relatively smaller than that for CuHL by a gap of about 0.8 pK unit, which facilitates the hydrolysis of PNPP.

Graphical Abstract Four orders of magnitude rate enhancement was observed for the catalytic hydrolysis of *p*-nitrophenyl picolinate (PNPP) by one μ -alkoxide/ acetato-bridged binuclear copper(II) complex under physiological conditions. Substrate specificity of the resulting binuclear complexes was observed for the hydrolysis of PNPP and AE-active ester.



Keywords Bimetallic cooperation · Artificial enzyme · Hydrolysis · Comparable kinetics

Introduction

Natural hydrolases containing multi-metal centers play vital roles in some physiological activity [1], which mainly involve in the selective and efficient cleavage of

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the esters, amides and DNA [2-4]. As noted in a number of previous researches, remarkable catalytic efficiency of hydrolases shall be ascribed to bimetallic synergetic effect, hydrogen bond activation and hydrophobic effect [5], and so on. Binuclear and poly-nuclear metal complexes are dominative candidates for metallohydrolases with enzymatic efficiency so far [6-13]. The main driving motivation is that related researchers devoted to elucidate the similar catalytic performances of those di- or polynuclear complexes used. Moreover, major interests were dedicated to simulating the structural features of catalytic sites and binding sites [14]. Functional mimics of hydrolases are highly important for understanding the role of metal ions in the hydrolytic mechanisms and developing artificial hydrolases. In varied designing strategies for the construction of multi-center moieties, bridging units (e.g., phenolate [15, 16], alkoxide [17], carboxylate [18], etc.) were widely serviced as key components for the bridging of metal ions.

In our previous study [19], it was also found that an alkoxo-bridged binuclear Cu(II) complex is more active than its analogue with solo-metal center for excess two orders of magnitude in the hydrolysis of scissile p-nitrophenyl picolinate (PNPP). Additionally, a phenoxobridged binuclear Cu(II) complex with amino acid Schiff base ligand exhibited two or three orders of magnitude rate enhancement in comparison with spontaneous hydrolysis of PNPP [20]. Inspired by structural properties of binuclear metalloenzymes, here we further investigated the reactivity of one bromo-containing μ -alkoxo/acetatobridged binuclear copper(II) complex (Cu₂L(OAc), Fig. 1) towards the PNPP hydrolysis. This case is comparable for the previous work concerning chloro-containing or nonhalogen catalysts [21]. Moreover, a mononuclear CuHL (part A of Fig. 1) was screened during its catalytic activity

as a frame of reference. To elucidate the structure-activity relationship of the two complexes, molecular simulations were performed by Gaussian program for comparing the different coordination sphere of the as-prepared copper(II) complexes. Calculated molecule structures confirm that the binding site of binuclear Cu₂L(OAc) is relatively open relative to that of CuHL, which leads to an easier formation of reactive substrate-catalyst binary complex. Probably taking account to possibly distinguishable selectivities and activities of the resulting catalysts towards different substrates, herein we conducted one control experiment concerning the hydrolysis of a selected active ester, i.e., S-2-benzothiazolyl 2-amino-alpha-(methoxyimino)-4-thiazolethiolacetate (AE-active ester) (part B of Fig. 1) which widely acts as raw material of ceftriaxone and claforan, mediated by the two copper(II) catalysts used in the present work.

Experimental

Materials and instruments

All reagents were of analytical grade and used as supplied, unless otherwise noted. 1,3-diamino-2-propanol, Tris-H⁺ [tris(hydroxymethyl) aminomethane], and potassium chloride were products from Sigma-Aldrich Co. Triethylamine was purchased from Chengdu Kelong Chemical Co. 5-Bromosalicylaldehyde was prior product in our lab. The ligand, namely 1,3-bis(5-bromosalicylideneamino)-propan-2-ol (H₃L) was synthesized according to the published procedure [22]. Binuclear Cu₂L(OAc) and mononuclear CuHL were prepared using the following modified methods described. Highly purified-water with resistance of excess 18 M Ω ,

Fig. 1 Schematic structures of the resulting copper (II) complexes (part A) and two substrates (part B)



which come from a Water Purification System (Nex Power 1000, Human Corporation, South Korea), was used throughout for kinetic runs. PNPP as substrate was prepared by a prior art [23]. S-2-benzothiazolvl 2-aminoalpha-(methoxyimino)-4-thiazolethiolacetate was purchased from J and K Scientific Ltd. Ionic strength (I) was maintained at a constant value (0.1 M) with KCl. The ¹H NMR spectrum was determined at 400 MHz on a Bruker Avance-400 NMR spectrometer (Bruke Corporation, Germany). Mass spectra of the two complexes were recorded on AGILENT 1100 LC-MS (Agilent, USA). Elemental analysis was carried out on CHNOS Elemental Analyzer Vario EL Cube (Germany Elementar Co.). Melting point determination was conducted on X-5-type Digital Precision Melting Point Apparatus (Beijing Fukai Instrument Co., Ltd., China). PNPP stock solution (0.02 M) was freshly prepared with distilled acetonitrile. Hydrolysis reaction of PNPP was detected by monitoring the appearance of the product *p*-nitrophenolate anion at 400 nm on a GBC Cintra 1010 UV-Vis spectrometer (Australia). Optimal structures of both copper(II) complexes were calculated by Gaussian 09 W software (Revision D.01).

Synthesis of 1,3-bis(5-bromo-2-salicylideneamino)-propan-2-ol (H₃L)

Schiff base ligand (H₃L) was synthesized by reaction of 1, 3-diamino-2-hydroxypropane and 5-bromosalicylaldehyde in 1:2 molar ratio in anhydrous ethanol as solvent at 75 °C. The mixture was stirred for 4 h, and cooled to room temperature. The Schiff base compound (H₃L) was obtained as yellow crystals after a recrystallization of the crude products with anhydrous methanol. H₃L: yellow crystals with a yield of 93.6%. m.p 161.2–161.8 °C. ¹H NMR(DMSO) δ : 13.66 (s, 2H, ArO*H*), 8.51 (s, 2H, N=CH), 6.83–7.68(m, 6H, Ar*H*), 5.26–5.27 (m, 1H, CH–CH₂), 3.32–3.80(m, 4H, NCH₂), 4.00(s, 1H, CH–OH); IR(KBr, film) ν_{max} : 3432, 3060, 2895, 1637, 1476, 1274, 807. Anal. calcd. For C₁₇H₁₆N₂Br₂O₃: C 44.76, H 3.54, N 6.14; found C 45.35, H 3.57, N 6.15%.

Synthesis of binuclear Cu₂L(OAc)

For the preparation of the binuclear copper(II) complex, a solution of 0.2 mmol Cu(OAc)₂·H₂O was prepared with 90 ml of methanol/water mixed solvent (5:1 v/v). After that 0.1 mmol Schiff base ligand solved in a 25 ml of methanol/ DMSO mixed solvent (4:1 v/v) was added into the resulting solution containing copper salt. The mixed solution was stirred for 2 h, and then allowed to evaporate at room temperature to give blue-green crystals, which were collected and washed with ethanol. Cu₂L(OAc): blue-green solid, yield of 91.51%. m. p. > 201.3 °C (dec.). IR(KBr, film) ν_{max} : 1636(C=N). m/z:

calc. for $C_{19}H_{16}Cu_2Br_2N_2O_5$ 639.80; found 640.81 (M + H), 662. 80 (M + Na). Anal. calcd. For $C_{19}H_{16}Br_2Cu_2N_2O_5$: C 35.70, H 2.52, N 4.38; found C 35.66, H 2.58, N 4.29%.

Synthesis of mononuclear CuHL

CuHL was prepared by the reaction of 1,3-bis(5bromosalicylideneamino)-propan-2-ol in a 25 ml mixed solvent of ethanol/DMSO (4:1 v/v) with 1 equiv. of $Cu(OAc)_2 \cdot H_2O$ solved in 40 ml of anhydrous ethanol. The solution of the copper(II) acetate was added dropwise into the solution of 1,3-bis(5-bromosalicylideneamino)-propan-2-ol. The reaction mixture was kept stirring for 1 h and then cooled at room temperature, yielding yellow-green powders as crude product. Yellow-green powders as end product was collected after further recrystallization of the crude products using anhydrous ethanol. CuHL: yellow-green solid, yield of 94.06%. m. p. > 260 °C (dec.). IR(KBr, film) v_{max} : 3396(O-H), 1621(C=N). *m/z*: calc. for C₁₇H₁₄CuBr₂N₂O₃ 516.86; found 517.85 (M + H), 539. 87 (M + Na). Anal. calcd. For C₁₇H₁₄CuBr₂N₂O₃: C 39.44, H 2.73, N 5.41; found C 39.88, H 2.74, N 5.41%.

Hydrolysis kinetics

Kinetic measurements of PNPP hydrolysis mediated by the resulting copper complexes were performed in a pH range 6.50–8.50. Pseudo first-order rate constants (k_{ob}) were determined by monitoring the absorbance changes of the released p-nitrophenolate anions (PNP) at 400 nm for the PNPP hydrolysis. Each kinetic run started with an injection of substrate stock solution into a preheated buffered solution containing catalyst. For comparison, at a temperature of 17 °C contrast kinetics of AE-active ester were carried out. Moreover, during the current control runs, the final concentration of AE-active ester was kept at a lower level (0.033 mM) because AE-active ester with a higher concentration is hardly soluble in the catalytic system. Hydrolysis rates of AE-active ester were determined by the detection of one hydrolytic product (2-mercaptobenzothiazole, abbr. 2-MBT) at 309 nm in buffered catalytic system containing the binuclear Cu₂L(OAc). The data reported are averages of three runs with uncertainty of less than 5%.

Results and discussion

Hydrolysis rates of PNPP catalyzed by binuclear and mononuclear copper(II) complexes

Owing to the unique characters and good performance of bimetallic complexes in contrast with their mononuclear analogues, many reports involved the design of binuclear metal complexes with various ligands [24–26]. Herein, we investigated the hydrolytic reactivity of a binuclear copper (II) complex (Cu₂L(OAc)) with an alkoxo-containing compound (H₃L). Shown in Fig. 2 are the pH-rate constant profiles of PNPP hydrolysis by Cu₂L(OAc) and the mononuclear CuHL. The observations denote that the binuclear Cu₂L(OAc) efficiently accelerated the hydrolytic cleavage of PNPP by a four-orders-of-magnitude rate enhancement under almost neutral conditions (25 °C, pH 7.0) compared to the background rate ($k_0 = 7.8 \times 10^{-6} \text{ s}^{-1}$) of PNPP spontaneous hydrolysis [23].

Figure 2 shows that binuclear Cu₂L(OAc) displays notable activity, giving rise to ca. 11031-fold rate enhancement against an un-catalyzed reaction [23]. Unfortunately, under comparable conditions the activity of the mononuclear one was less active than binuclear one, Cu₂L(OAc). Namely, CuHL acted as a poor catalyst only showing about 90-fold rate enhancement in comparison with the spontaneous hydrolysis of PNPP. The above-mentioned facts provide evidence for the bimetallic cooperation of the two copper ions, which are highly in agreement with previous reports [19, 27]. Furthermore, the rate constants for the Cu₂L(OAc)-induced PNPP hydrolysis exhibit bell-shaped curves as a function of pH, which has enzyme-like behavior [28]. The best catalytic efficiency reached around pH 7.6, followed by a sharp decrease in rate constant with further increases in the pH value. This feature has an inseparable relationship with the pH-dependent deprotonation of the metal-bound water. As compared to that for binuclear complex, a sigmoid-shaped changing tendency of hydrolysis rates is represented for the CuHL-promoted hydrolysis of PNPP. This observation implies that the maximum rate for the PNPP hydrolysis induced by CuHL probably appears only at above pH 8.50. Above changing tendency is almost the same as in Fig. 1S that showed the pH-respondant variation of initial hydrolysis rate. Observations mentioned above consists the pH-respond features of natural enzyme possessing its own optimum range for pH where it will be most active.

To confirm if the prepared copper complexes still possess good activity in the catalytic hydrolysis of the other esters, herein we comparatively evaluated the hydrolytic reactivity of binuclear copper complex towards the hydrolysis of PNPP and AE-active ester as a contrast model substrate (Fig. 3) in the operated pH range (6.50–8.50). It was found that binuclear copper complex showed its better catalytic function in the hydrolysis of the two substrates used here. This result may be ascribed to good-activity of Cu₂L(OAc) which results from the bimetallic synergistic effect. Also, in term of both substrates Cu₂L(OAc) represents enzyme-like bell-shape pH response. Thereupon, Cu₂L(OAc) is considered as a potential mimic model of natural carboxylases.

Additionally, under the same concentration (0.033 mM) hydrolysis performance of AE-active ester and PNPP by $Cu_2L(OAc)$ and CuHL was conducted at selected pH (7.30 and 7.60), for evaluating their distinguishable activities of the two complexes. Experimental observations provides us with two important information concerning substrate selectivity and bimetallic cooperation (Fig. 4). That is, binuclear $Cu_2L(OAc)$ displayed



Fig. 3 pH-dependent rate constants of the hydrolysis of PNPP and AE-active ester induced by Cu₂L(OAc) in the range of 6.50–8.50. Conditions: 17 ± 0.1 °C, [PNPP]_{final} = 3.3×10^{-5} mol L⁻¹, [complex] = 1×10^{-5} mol L⁻¹

Fig. 2 pH-dependent first-order rate constants of the PNPP hydrolysis by Cu₂L(OAc) (*left*) and CuHL (*right*) in the PNPP concentration range of 0.200–0.467 mM. Conditions: 25 ± 0.1 °C, [PNPP]_{stock} = 2×10^{-2} mol L⁻¹, [complex] = 1×10^{-5} mol L⁻¹





Fig. 4 Relative hydrolytic rate of PNPP and AE-active ester induced by Cu₂L(OAc) and CuHL at selected pH values, i.e., 7.30, 7.60. Conditions: 17 ± 0.1 °C, [PNPP]_{final} = 3.3×10^{-5} mol L⁻¹, [AE-active ester]_{final} = 3.3×10^{-5} mol L⁻¹, [complex] = 1×10^{-5} mol L⁻¹

about 1.23-fold or 1.32-fold kinetic advantage for the PNPP hydrolysis in contrast with AE-active ester. However, CuHL-promoted hydrolysis of AE-active ester is approximately 4.43-fold or 5.78-fold faster than that of PNPP, suggesting CuHL possesses much better selectivity in the hydrolysis of AE-active ester. Another aspect represents that binuclear one is indeed a better catalyst to accelerate the hydrolysis process of both substrates used in this work. Moreover, Cu₂L(OAc) showed more obvious advantage (about 50 or 68 time) compared to CuHL for accelerating PNPP hydrolysis, whereas in the case of hydrolysis of AE-active ester, declined activity difference between both catalysts was detected revealing about ninefold difference in catalytic activity. In short, CuHL is more efficient for catalyzing hydrolysis of AE-active ester, and $Cu_2L(OAc)$ is able to play a more positive role in accelerating hydrolysis of PNPP. This property provides evidence for the substrate specificity of natural hydrolases [29-31], which confirms that the artificial enzymes are required for favorable intrinsic structures so that it match various substrates.

Distinguishable structure–mechanism relationship of PNPP hydrolysis by di- or mononuclear complexes

In the field of artificial metallohydrolases, it is widely accepted that the metal-induced hydrolysis processes of esters involve in the nucleophilic attack of the active metal-OH species on the substrate bound to the central metal [32]. In the metal-mediated hydrolytic process, there are at least three aspects of contributions of the central metals [33, 34], which concerns (1) the binding activation of substrate; (2) the activation of metal-bound H_2O ; (3) the stabilization of the tetrahedron transition state. As for our present study, the divalent copper cores of the as-prepared copper(II) complexes also play a similar role in the hydrolysis of PNPP as a typical model of carboxylic acid esters.

Part A of Scheme 1 shows a possible mechanism of the PNPP hydrolysis promoted by Cu₂L(OAc) during which there may exist two kinds of hydrolysis modes. That is, the PNPP hydrolysis occurs via a nucleophilic attack by H₂O bound to Cu(II) ion (not shown here) and its deprotonated Cu(II)-hydroxyl form. As the operated pH is below the systematic pK_{a1} of the bound H₂O for Cu₂L(OAc), lower reaction rates were observed due to the worse nucleophilicity of bound water relative to nucleophilic reactivity of the Cu(II)-linked hydroxyl forms. In contrast, sharp increase in rate constant (see Fig. 2) appears when the operated pH exceeds its inherent pK_{a1} . In the hydrolytic system, dihydrate complex undergoes a two-step deprotonation in which an active one-hydrated Cu(II)-OH⁻ species is involved. Afterwards, PNPP as substrate then links to another Cu(II) core through a displacement of bound H₂O with PNPP. Efficient association of substrate to the binding site of an enzyme is crucially important for gaining remarkable efficiency due to a declined energy barrier and shortened distance of PNPP to the catalytic nucleophiles [1]. Subsequently, an intramolecular nucleophilic attack of the active Cu(II)-OH- on the carboxyl of PNPP releases a detectable chromophore (p-nitrophenolate anion, pNP) which originates from the deprotonation of *p*-nitrophenol in the operated pH region. Negative tetrahedron transition state (TS) can be stabilized by the positive copper(II) ion, which is beneficial to the rate acceleration. Lastly, dihydrated species as the catalyst precursor is regenerated by the displacement of picolinic acid with free water, arousing new hydrolytic cycle. By the way, an obvious declining of rates shall be contributed to the formation of inactive dihydroxide form when the operated pH beyond the pK_{a2} value.

In spite of a similar catalytic pathway for the PNPP hydrolysis catalyzed by Cu₂L(OAc) and CuHL (part B of Scheme 1), there are distinguishable properties between both the hydrolytic systems containing different copper complexes due to their different structures. Bimetallic sites of Cu₂L(OAc) lead to a favorable catalytic mode of the PNPP hydrolysis. First of all, the corresponding transition state is relatively stable owing to smaller steric repulsion for the Cu₂L(OAc)-bearing system. Moreover, the two Cu(II) ions of $Cu_2L(OAc)$ can efficiently act as the binding site and the catalytic site, displaying a positive cooperative bimetallic effect. As a result, Cu₂L(OAc) is a highly active catalyst for the hydrolysis of PNPP. In the case of CuHL, however, both substrate (PNPP) and water molecule bound to the same one copper ion, resulting in higher activation energy (E_a) due to the bigger steric repulsion, as well as



Scheme 1 Proposed mechanisms of the $Cu_2L(OAc)$ -promoted hydrolysis of PNPP. Part B only gives the formation of reactive PNPP-CuHL binary complex and the tetrahedral transition state for clarity

a difficult *cis*-orientation controlling nucleophilic hydroxide anion to substrate (Scheme 1). Thus, the CuHL-induced PNPP hydrolysis is less reactive in comparison with $Cu_2L(OAc)$.

The spectral studies (Fig. 5) were performed for grasping information about the hydrolytic routes of PNPP mediated by Cu₂L(OAc) and CuHL, respectively. New strengthened spectra marked with PNPP-complex (Cu₂L(OAc) or CuHL) denote the formation of the reactive $Cu_2L(OAc)$ -PNPP binary complexes. As the reaction time was enlarged, one can find that the absorbance at 263 nm gradually decreased suggesting the consumption of PNPP. Meanwhile, the increased absorption at 400 nm indicates the formation of *p*-nitrophenolate anions (*pNP*) as one of the products. Also, two inserted pictures in Figs. 5a and c showed distinguishable yellow with respect to the concentration of the producing *p*-nitrophenolate anion at the same time interval, providing direct evidence for the much better activity of Cu₂L(OAc) in comparison with CuHL. Furthermore, the distinguishable spectra of the two copper(II) complexes were obtained in absolute ethanol and aqueous solution, respectively. Namely, the absorption spectra showed in Figs. 5a and d represents approximately 2.4 and 4.8 nm red shifts for Cu₂L(OAc) and CuHL in aqueous solution in contrast with those in absolute ethanol. The above-mentioned changed absorptions to some extent confirm the formation of dihydrated complexes, $Cu_2L(H_2O)_2$ or $CuHL(H_2O)_2$. In short, UV–Vis spectral scans shown in Fig. 5 demonstrate that the proposed mechanism of PNPP by the prepared copper complexes is reasonable and reliable.

Two characteristic absorption bands of the formed 2-MBT, which originated from the hydrolysis of AE-active ester, respectively, locate at wavelength range of 230–240 and 308–320 nm [35]. As for the AE-active ester hydrolysis mediated by Cu₂L(OAc), strengthened absorption (see Fig. 6) located at 230–240 nm indicates the formation of binary catalyst–substrate complex, which is highly in accordance with the generation of PNPP–Cu₂L(OAc) intermediate (Fig. 5a). Moreover, UV scans show the sustained increase in the strength (orange arrow) of both characteristic peaks of 2-MBT, companying with gradual decrease (green arrow) in the concentration of AE-active ester. Changed absorption mentioned above suggests that Cu₂L(OAc) also efficiently catalyzes the hydrolysis cleavage of AE-active ester besides PNPP.

The kinetic parameters including catalytic constant (k_{cat}) and binding constant (K_s) were determined by the

Fig. 5 UV–Vis spectra (a and c) for the hydrolysis of PNPP by $Cu_2L(OAc)$ or CuHL. In addition, the absorption spectra (b and d) of the two copper complexes was recorded in the water and absolute ethanol, respectively



corresponding slopes and intercepts of the double-reciprocal plots in Fig. 7. The catalytic constant k_{cat} (1111 s⁻¹) for the Cu₂L(OAc)-induced PNPP hydrolysis is about 1.8 times than that (625 s^{-1}) for the CuHL-promoted hydrolysis of PNPP. At the same time, the linkage of PNPP to binuclear Cu₂L(OAc) is much easier than that of PNPP to CuHL, which can be supported by a 34-fold value (0.69 M^{-1}) of binding constant compared to the latter (0.02 M^{-1}) . According to enzymology theory, the formation of substrate-catalyst binary complex intermediate highly facilitates the acceleration of enzyme reactions [36]. Calculated total efficiency $(k_{cat}K_s)$ of Cu₂L(OAc) towards the PNPP hydrolysis at pH 7.6 is approximately 769.2 s⁻¹·M⁻¹, exhibiting 61-fold kinetic advantage against CuHL. Hence, in our case the high rates for the Cu₂L(OAc)-catalyzed hydrolysis of PNPP is partially ascribed to the relatively convenient generation of reactive Cu₂L(OAc)-PNPP intermediates. The easier linkage of PNPP to Cu₂L(OAc) shall be contributed to the intrinsic structure of Cu₂L(OAc), which is confirmed by the optimal geometry shown in the later section.

Feasible methodology for the determination of pK_a value of Cu(II)-bound H₂O

Metal-bound OH⁻ species are the real active form of nucleophilic attack on the substrate in the hydrolysis process of esters. Meanwhile, the generation of this active species is relevant to the systematic acid–base circumstance. Appropriate pH is thereby required for natural hydrolases to maintain the optimum enzyme efficiency and keep the majority of enzymes from deactivation [37]. Conversely, extreme pH condition may cause extensive deactivation of enzymes due to a negative pH-dependant allosteric structure.

In the hydrolysis of phosphate esters and carboxylates, it is expected that the formation of the active metal-hydroxyl species with a high concentration level at enzyme's optimum pH. According to the proposed mechanism (see Scheme 1), there is a two-step deprotonation process of the dihydrate copper(II) complex. The first deprotonation of the one water bound to the divalent Cu(II) core produces the Cu(II)-OH⁻ species with a dissociation constant, K_{a1} . Furthermore, the ionization of another bound H₂O to the second central copper happens as the systematic pH excesses its intrinsic dissociation constant (K_{a2}) . In the current work, Cu₂L(OAc) displays good activity under almost neutral condition, whereas CuHL is proved to be a worse catalyst. This fact shall be traced to two main reasons. Apart from the easier linkage of PNPP with the binding site of Cu₂L(OAc), the formation of the nucleophilic metalhydroxide species in the Cu₂L(OAc)-containing system is easier than that in another system in the presence of CuHL under comparable conditions.







Fig. 7 Linear curve fitting of k_{ob}^{-1} -[PNPP]⁻¹ for the hydrolysis of PNPP by Cu₂L(OAc) or CuHL, respectively. Conditions are the same as in Fig. 2

Herein, we attempt to adopt a second derivative method (SDM) [38] acted as an applicable and convenient one for the determination of pK_{a1} of bound H₂O. Figure 8 gives the respective second derivatives profiles responding to k_{ob} -pH

curves for the two copper complexes-catalyzed hydrolysis of PNPP. For each substrate concentration level, the desired pK_{a1} can be obtained by the corresponding pH value which corresponds to the second derivative with a zero value. That is to say, each value of pK_{a1} can be determined by the *x*-axis value of the intersection point of the depicted zero baseline and each second derivative curve. Table 1 summarizes all of pK_{a1} values determined with SDM.

The data shown in Table 1 give us at least two aspects of information. The one is that the central copper(II) ion efficiently activates the bound water with a lower pK_{a1} value, which benefits the nucleophilic attack of the Cu(II)bound hydroxyl on the PNPP under weak-basic condition. Another one is that binuclear Cu₂L(OAc) possesses more powerful ability to lower the pK_{a1} of the bound water in contrast with CuHL. The pK_{a1} value of the H₂O bound to Cu₂L(OAc) around 7.20, and the pK_{a1} of CuHL-bound H₂O is about 8.0. Notably, the pK_{a1} values of bound water in Table 1 were found to be almost coincident, regardless of CuL(OAc) or CuHL demonstrating that the deprotonation process of the bound H₂O prior to the linkage of PNPP with metal complex. This experimental evidence

| [PNPP] (mM) | pK_{a1} values of the Cu(II)-bound H_2O | |
|-------------|---|------|
| | Cu ₂ L(OAc) | CuHL |
| 0.200 | 7.20 | 7.98 |
| 0.267 | 7.16 | 7.99 |
| 0.333 | 7.15 | 7.99 |
| 0.400 | 7.15 | 7.97 |
| 0.467 | 7.15 | 7.98 |

Table 1 Determined pK_{a1} values of the bound water using second derivative method

Conditions are the same as in Fig. 2

strongly supports the proposed mechanism shown in Scheme 1. Indeed, one can draw a conclusion that the difference in pK_{a1} of 0.8 pK unit probably acts as a crucial factor for highly prompting the catalytic activity of Cu₂L(OAc).

Structure–activity relationship of the As-prepared copper(II) complexes

Those researches concerning the simulation of natural metalloenzymes provide invaluable resources for designing and synthesizing more novel and efficient artificial enzymes [39]. Especially, poly-nuclear metal complexes containing oxygen-bridged copper(II) are of particular interest because they might represent valuable model compounds for the active site of a number of metalloenzymes [40].

In our work, binuclear Cu₂L(OAc) is found to be more reactive than its mononuclear analogue, CuHL. Under the same conditions (pH 7.0, 25 °C), the relative reactivity of Cu₂L(OAc) to CuHL is up to 122.9. Additionally, optimum efficiency of Cu₂L(OAc) is achieved at pH 7.6, while that of CuHL probably reaches at pH > 8.20. This fact shall be attributed to their different structures of Cu₂L(OAc) and CuHL. The two oxygen-bridged copper moieties of the former are close to each other, resulting in an appropriate Cu(II)...Cu(II) distance (~3.6Å, a calculated distance from molecule structure optimization shown in Fig. 2S) for acting as the catalytic site and binding site of metallohydrolases. This is in accordance with the feasible metalmetal distance (3.0–4.0 Å) of natural metalloenzymes [41]. Hence, positive bimetallic synergic effect [42] plays a vital role in the hydrolysis of PNPP and AE-active ester mediated by $Cu_2L(OAc)$, leading to dominated reactivity of $Cu_2L(OAc)$ against CuHL.

Based on the following equation, $pK_a = -\log(K_w/$ [H₂O]), in which K_w is about $1.0 \times 10^{-13.79}$ [43, 44] and [H₂O] equals to 55.5 M, the determined pK_a of free water at 0.1 M ionic strength (KCl) is about 15.5 at 25 °C. As a result, the high pK_{a} (15.5) reveals the highly weak deprotonation of water. For hydrolysis reactions of some compounds including esters mediated by various metallohydrases, the metal-bound hydroxide forms shall be the best nucleophilic reagent rather than metal-bound water and free water. Therefore, it is a challenge that the designs of artificial metalloenzymes with high activity and ability of activating the bound water. In the present study, $Cu_2L(OAc)$ gives an obvious pK_a declining by about eight pK units relative to that of free H_2O , which is in favor of the nucleophilic attack of PNPP by the Cu(II)-OH⁻ species even under physiological conditions (pH 7.0, 25 °C). At some extent $Cu_2L(OAc)$ is able to mimic the binuclear metal centers of natural metalloenzymes. As the result of those factors mentioned above, it is comprehensible that binuclear Cu₂L(OAc) displays better activity than CuHL in the hydrolysis of PNPP.

By the way, we can find that the catalytic activity of the bromo-containing Cu₂L(OAc) in this case is relatively lower in comparison with that analogue [21] with chloride substituents. Two aspects of factors should be considered. The one may be that the much bigger steric hindrance of bromine group blocks the association of PNPP with the central copper, which is negative for the PNPP hydrolysis. Another one is that the deprotonation of the bound water on the copper site of bromo-containing Cu₂L(OAc) is more difficult than that of chloro-bearing analogue, which can be confirmed by the bigger pK_{a1} (~7.2) of the bound water on the former compared to the latter ($pK_{a1} = 7.0$). This phenomenon probably results from the stronger

Fig. 8 Profiles of the second derivatives of k_{ob} values as a function of pH for the Cu₂L(OAc)- and CuHL-containing systems, respectively





Fig. 9 pH-respondant relative rates of the hydrolysis of PNPP by Cu₂L(OAc) and CuHL in the PNPP concentration range of 0.200–0.467 mM. Conditions: 25 ± 0.1 °C, [PNPP]_{stock} = 2×10^{-2} mol L⁻¹, [complex] = 1×10^{-5} mol L⁻¹

electron-withdrawing property of the chlorine atom in contrast with bromine atom, resulting in the increasing Lewis acidity of the central copper inside the chloro-bearing binuclear complex.

Figure 9 shows the relative values of the hydrolysis rate constants of PNPP by $Cu_2L(OAc)$ and CuHL. It can be observed that an outstanding activity difference between the two complexes in almost neutral environment (pH 7.0). For instant, it was observed that about 123-fold difference in activity between the two copper(II) complexes at pH 7.0, [PNPP] = 0.2 mM. This behavior is the result of much better activity of $Cu_2L(OAc)$ and the worse activity of CuHL at the comparable pH (7.0). On the contrary, the declining difference in reactivity between both copper complexes shall be attributed to the decreasing activity of $Cu_2L(OAc)$ and the increasing activity of CuHL as pH continues to increase (refer to Fig. 2).

Conclusions

In summary, alkoxide/acetato-bridged binuclear copper(II) complex $Cu_2L(OAc)$ exhibits prominent reactivity in the PNPP hydrolysis reaction, announcing four-orders of magnitude rate enhancement under physiological conditions. Moreover, bell-shaped pH-respond curves of hydrolysis rate demonstrate that the binuclear $Cu_2L(OAc)$ is an ideal hydrolase model. The main reasons might be the positive cooperative bimetallic roles of the two central copper(II) cores on the binding of substrate, activation of bound H₂O and substrate. Accordingly, the resulting bromo-containing $Cu_2L(OAc)$ is shown to be a very efficient PNPP hydrolysizing agent. At the same time, $Cu_2L(OAc)$ has excellent performance in the catalytic hydrolysis of AE-active ester, a kind of raw material for a few medicines (ceftriaxone and claforan). On the contrary, mononuclear CuHL proves to be not a good catalyst for the PNPP hydrolysis due to its instinctive structure. Interestingly, CuHL exhibited much better activity towards AE-active ester hydrolysis in contrast to PNPP. These facts highly testify that higher substrate-catalyst matching level is beneficial to the reaction acceleration. Our research will impel the construction of novel mimic enzymes with various bridged units. Structural optimum confirms the more open binding site of Cu₂L(OAc) that results in easier linkage of PNPP with Cu₂L(OAc). In addition, we prospect that the introduction of more eletron-withdrawing substituent groups into the ligand molecules should upgrade the activity of designed artificial metallohydrolases.

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