

Artificial Trinuclear Metallopeptidase Synthesized by Cross-Linkage of a Molecular Bowl with a Polystyrene Derivative

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Abstract: A novel methodology is reported for construction of active sites of artificial multinuclear metalloenzymes: transfer of metal-chelating sites confined in a prebuilt cage to a polymeric backbone. Artificial active sites comprising two or three moieties of Cu(II) complex of tris(2-aminoethyl)amine (tren) were prepared by transfer of Cu(II)tren units confined in a molecular bowl (MB) to poly(chloromethylstyrene-co-divinylbenzene) (PCD). By treatment of unreacted chloro groups of the resulting PCD with methoxide and destruction of the MB moieties attached to PCD with acid followed by addition of Cu(II) ion to the exposed tren moieties, catalytic polymers with peptidase activity were obtained. The average number (β) of proximal Cu(II)tren moieties in the active site of the artificial multinuclear metallopeptidase was determined by quantifying the Cu(II) content. Several species of the artificial metallopeptidases with different β contents were prepared and examined for catalytic activity in hydrolysis of various cinnamoyl amide derivatives. The PCD-based catalytic polymers did not hydrolyze a neutral amide but effectively hydrolyzed carboxyl-containing amides (*N*-cinnamoyl glycine, *N*-cinnamoyl β -alanine, and *N*-cinnamoyl γ -amino butyrate). Analysis of the kinetic data revealed that the active sites comprising three Cu(II)tren units were mainly responsible for the catalytic activity. When analyzed in terms of k_{cat} , the catalytic activity of the PCD-based artificial peptidase was comparable to or better than the catalytic antibody with the highest peptidase activity reported to date. A mechanism is suggested for the effective cooperation among the three metal centers of the active site in hydrolysis of the carboxyl-containing amides.

Introduction

Proteins have been chosen as the backbone of enzymes by nature, since only large macromolecules can carry enough molecular information both for substrate recognition and thermodynamic efficiency of chemical transformation that are needed for effective catalysis of biochemical reactions.¹ Likewise, macromolecules have been employed as the skeletons of artificial enzymes such as catalytic antibodies^{2–5} and catalytic polymers.^{6–10} Nature has improved the efficiency of its own catalytic system built on polypeptides. Thus, it is unlikely to obtain artificial enzymes as effective as natural enzymes with respect to catalytic rates or selectivity by using polypeptides as the backbone of artificial enzymes. Moreover, it is difficult to achieve stability to heat and compatibility with organic solvents when polypeptides are used as the backbone of artificial enzymes. In this regard, synthetic polymers are useful as an alternative backbone of effective and stable artificial enzymes.

At present in the study of catalytic antibodies or catalytic polymers, major efforts are being made in the development of

new strategies for designing active sites. Whether the strategy is successful is judged by the activity of the artificial enzymes produced, although the structure of the active site may not be well characterized and the mechanism of catalysis may not be clearly understood on the molecular level. For designing active sites comprising multiple number of catalytic groups on synthetic polymers, a few methodologies have been reported. For example, we have developed several new methods such as attachment of both binding and catalytic sites, site-directed functionalization, cross-linkage of catalytic elements with a macromolecular spacer, and self-assembly from catalytic elements.^{10–14}

Many enzymes contain two or more metal ions in the active site, exploiting collaboration among the metal centers in catalytic conversion of complexed substrates. Examples of multinuclear metalloenzymes catalyzing hydrolysis of acyl derivatives and related compounds are methionine aminopeptidase,¹⁵ metallo- β -lactamase,¹⁶ proline dipeptidase (prolidase),¹⁷ urease,¹⁸ and agmatinase.¹⁹ In addition, there are a large number of multinuclear metalloenzymes that catalyze several other types of

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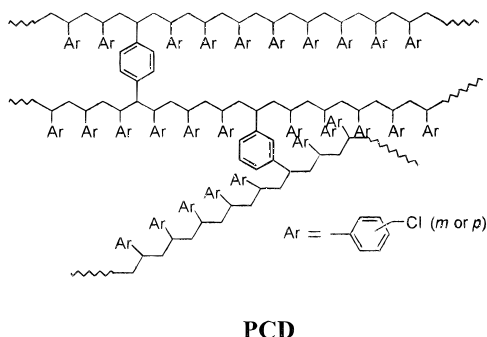
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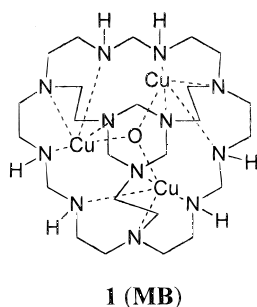
reactions such as nucleic acid hydrolysis, synthetic transformations, or oxidation–reduction.

An effective artificial enzyme would be obtained if an artificial active site comprising two or more proximal metal centers is designed. Various small molecules with multiple metal centers have been synthesized and tested for catalytic activity in several organic reactions including phosphoester hydrolysis²⁰ and asymmetric organic reactions such as epoxidation,²¹ aldol condensation,²² and carbonyl reduction.²³ No systematic efforts, however, have been made to design an artificial active site comprising multiple metal centers on the backbone of synthetic polymers. Here, we report construction of artificial multinuclear metalloenzymes through transfer of metal-chelating sites confined in a prebuilt cage. The catalytic metallopolymer thus obtained manifests high catalytic activity in amide hydrolysis as well as substrate selectivity.

The insoluble polymer backbone employed in the present study is poly(chloromethylstyrene-*co*-divinylbenzene) (PCD),¹³



a cross-linked polystyrene in which all of the styryl moieties contain chloromethyl groups. PCD possesses a high specific area and a high mechanical strength. In addition, it has a highly branched structure and plenty of reactive sites which are needed for introduction of multiple catalytic elements in proximity on the polymer skeleton. In the present study, two or three moieties of tris(2-aminoethyl)amine (tren) are positioned in proximity on PCD by using a trinuclear macrocyclic Cu(II) complex (**1**)



in which three moieties of Cu(II)tren are confined. Macrocyclic complex **1** is called a molecular bowl (MB) because of the bowl-like shape (**2**).

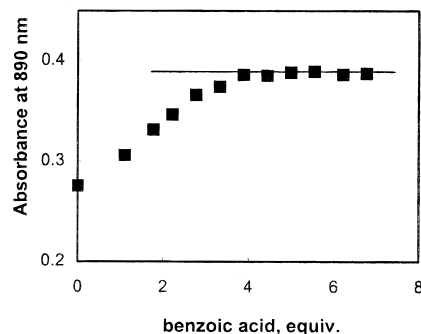
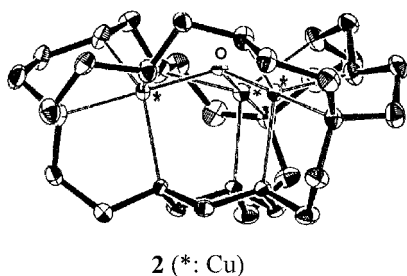


Figure 1. Absorbance changes observed upon addition of benzoic acid (0.149 M) to a solution of MBⁿ⁻ (1.08 mM) in DMF at 25 °C.

Results

Synthesis and Characterization of Catalysts. MB was synthesized through Cu(II)-template condensation of tren and formaldehyde by a one-pot reaction as reported previously.²⁴ Since MB contains six ionizable N–H bonds as well as two hydrate water molecules, ionization behavior of MB was examined. MB anion (MBⁿ⁻) was generated by treatment of MB with excess NaH in *N,N*-dimethyl formamide (DMF) and the subsequent removal of the unreacted NaH powder by filtration. In Figure 1, the absorbance change at 890 nm observed upon addition of benzoic acid to a solution of MBⁿ⁻ is illustrated. Only MBⁿ⁻ can cause the absorbance change upon treatment with acid, but a certain form of MBⁿ⁻ may not change the absorbance upon protonation. Addition of more than 4 equiv of acid does not affect the absorbance. Thus, the average size of negative charge on MBⁿ⁻ is at least 4 unless the basicity of hydroxide ion formed from the two hydrate water molecules is almost the same as that of MBⁿ⁻.

Synthetic route for the catalysts is summarized in Scheme 1. Upon attachment of MBⁿ⁻ to PCD, [MB]_αPCD is formed. Here, subscript α indicates the content of the MB site thus formed on [MB]_αPCD and is expressed in terms of % mol fraction relative to the styryl moieties of PCD. Treatment of [MB]_αPCD with sodium methoxide converts the chloromethyl groups of PCD to methoxymethyl groups, producing [MB]_αPCD^{MeO}. When [MB]_αPCD^{MeO} is treated with acid, Cu(II) ions dissociate from the MB site and the diaminomethyl units degrade, producing [(tren)_β]_αPCD^{MeO}. By quantification with inductively coupled plasma-absorption emission spectroscopy (ICP-AES) of the Cu(II) ion contained in the filtrate after treatment of [MB]_αPCD^{MeO} with HCl, the value of α is calculated. The diaminomethyl group stabilized by coordination to the metal ion in the framework of macrocyclic complex readily eliminates formaldehyde, producing two amino groups due to its intrinsic instability.²⁵ Although MBⁿ⁻ with *n* (the number of negative charge) of 3 is indicated in Scheme 1, *n* may be larger than 3. It should be noted that Scheme 1 represents the idealized situation that the three anions are symmetrically distributed in MB³⁻. It is also possible that one tren unit contains two anionic nitrogens which lead to a double fixation of tren to the polymeric backbone.

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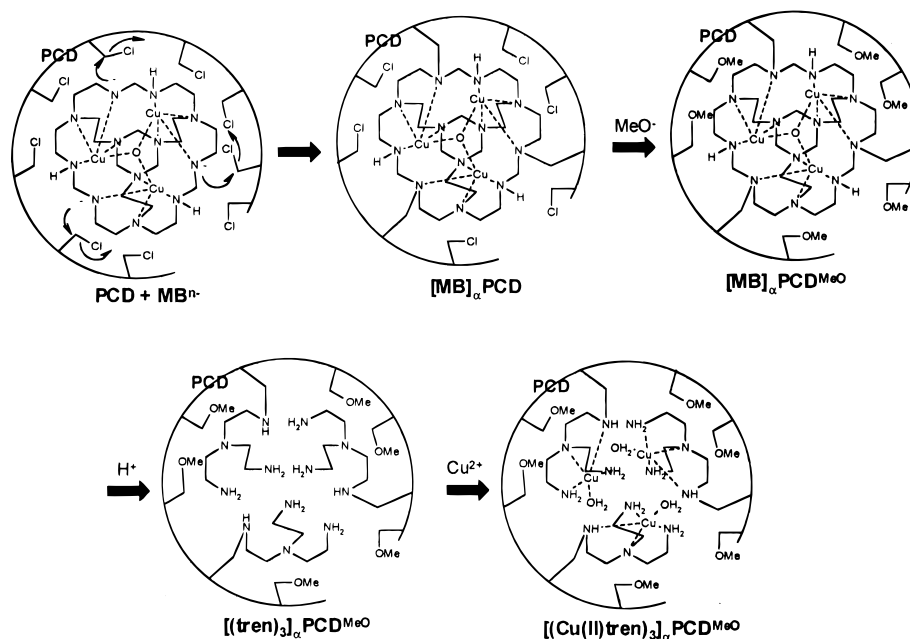
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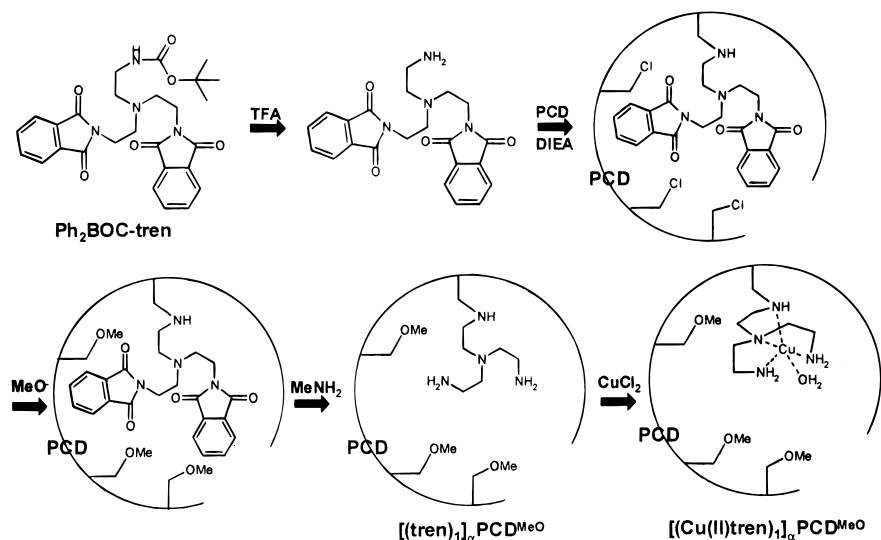
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Scheme 1



Scheme 2



If the nitrogen atom of a tren moiety of MB^{n-} is not connected to the resin during the formation of $[\text{MB}]_{\alpha}\text{PCD}$, that particular tren would dissociate from the resin upon treatment of $[\text{MB}]_{\alpha}\text{PCD}^{\text{MeO}}$ with acid. Then, only one or two tren moieties can be left after disassembly of an MB site. The average number of tren units per MB site obtained after disassembly of the MB site is indicated as subscript β ($1 \leq \beta \leq 3$). The particular active site illustrated in Scheme 1 contains three tren residues ($\beta = 3$). Upon addition of Cu(II) ion to $[(\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}}$, the tren moieties are converted to the Cu(II) complexes, producing $[(\text{Cu}(\text{II})\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}}$ which is transformed back to $[(\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}}$ upon treatment with HCl. By quantification with ICP-AES of the Cu(II) ion contained in the filtrate after treatment of $[(\text{Cu}(\text{II})\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}}$ with HCl, the value of β is calculated: $\beta = 3 \times (\text{amount of Cu released from } [(\text{Cu}(\text{II})\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}}) / (\text{amount of Cu released from the corresponding } [(\text{tren})_{\beta}]_{\alpha}\text{PCD}^{\text{MeO}})$

In some preparations, *p*-nitrobenzoic acid was added to MB^{n-} in various proportions to reduce the number of nitrogen anions available for attack at PCD and, thus, to lower the β value.

Four different catalysts were prepared by varying the amount of MB and by adding different amount of *p*-nitrobenzoic acid (0, 50, 100, and 200 mol %, respectively, relative to MB^{n-}): $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$, $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$, $[(\text{Cu}(\text{II})\text{tren})_{1.43}]_{1.7}\text{PCD}^{\text{MeO}}$, and $[(\text{Cu}(\text{II})\text{tren})_{1.11}]_{0.73}\text{PCD}^{\text{MeO}}$. The chemical yield for attachment of MB to PCD backbone was 20–74% as calculated from the α value, depending upon the amount of *p*-nitrobenzoic acid added to MB^{n-} . The yield decreased when more *p*-nitrobenzoic acid was used.

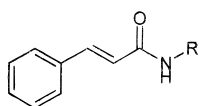
By using a doubly protected derivative of tren, a PCD resin ($[(\text{Cu}(\text{II})\text{tren})_1]_{1.7}\text{PCD}^{\text{MeO}}$) containing fully separated Cu(II)tren units was prepared as summarized in Scheme 2.

The relative amounts of Cl and Cu on the surface of $[\text{MB}]_{\alpha}\text{PCD}^{\text{MeO}}$ were estimated by Electron Probe Micro Analysis (EPMA), revealing that less than 2% of Cl atoms of chloromethyl groups originally present on the surface of PCD were left after treatment of $[\text{MB}]_{\alpha}\text{PCD}$ with sodium methoxide. The presence of primary amines in the tren-containing PCD derivatives was confirmed by Kaiser test using ninhydrin which forms Ruhemann's purple upon reaction with primary amines.²⁶

Quantification of the primary amines by Kaiser test was not, however, possible due to adsorption of Ruhemann's purple onto the resin. Instead, the content of the tren units was quantified by ICP-AES measurement of Cu ion released by acid treatment of Cu(II)tren-containing resins. Elemental analysis of the tren-containing PCD derivatives did not produce reliable estimates for the amount of nitrogen atoms in the resins presumably due to the presence of DMF adsorbed onto the resin during the preparative steps.

The formation constant (K_f) for Cu(II)tren moieties linked to PCD can be measured by assuming that binding of Cu(II) to a tren unit is independent of succeeding bindings by analogy with the Langmuir isotherm.¹³ By using the method described previously,^{13,27} $\log K_f$ was measured by a competition experiment using ethylenediaminetetraacetate (EDTA) as the chelating reagent of Cu(II) ion. Data for formation of Cu(II)EDTA by equilibration between EDTA and [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO} are illustrated in Figure 2. From the equilibrium constant estimated from nonlinear regression of the data and the formation constant for Cu(II)EDTA reported²⁸ in the literature, the average value of $\log K_f$ for the Cu(II)tren units in the resin was calculated. The values of $\log K_f$ thus obtained were 16.15 ± 0.09 for [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO} and 16.26 ± 0.07 for [(Cu(II)tren)_{1.1}]_{1.7}PCD^{MeO} at 25 °C and pH 8.00. Based on the parameters reported in the literature,²⁸ $\log K_f$ is calculated as 14.69 at 25 °C and pH 8.00 for the Cu(II) complex of tren unattached to polymers.

Amide Hydrolysis by Catalysts. The catalytic activity of [(Cu(II)tren) _{β}] _{α} PCD^{MeO} was examined by using amides **3–6** as the substrate. Hydrolysis of the neutral amide (**3**) was not detected when **3** was stirred with the PCD derivatives for 48 h at pH 8 and 50 °C. On the other hand, the carboxyl-containing amides (**4–6**) were hydrolyzed effectively by the PCD derivatives except [(Cu(II)tren)_{1.1}]_{1.7}PCD^{MeO}. When **4–6** were hydrolyzed by the catalysts, the quantitative formation of cinnamic acid was confirmed by HPLC analysis of the reaction product and that of the amino acids (glycine, β -alanine, and γ -aminobutyric acid) was confirmed by spectrofluorometric analysis using 2,3-naphthalenedicarboxaldehyde²⁹ as the coloring reagent.



- 3:** R = CH₃
4: R = CH₂COOH
5: R = CH₂CH₂COOH
6: R = CH₂CH₂CH₂COOH

Kinetic data for the hydrolysis of **4–6** catalyzed by various PCD derivatives were measured at 50 °C. In the present study, catalyst (C) concentration is taken as the concentration of the active site obtainable when it is assumed that the resin is dissolved in the buffer solution. The active site is defined as the site (MB site) originally occupied by MB on the PCD resin. The concentration of the amino acid released by amide hydrolysis was measured at various time intervals, from which the pseudo-first-order rate constant (k_o) was estimated. A typical example of the kinetic data used for evaluation of k_o is illustrated in Figure 3.

The pH profile of k_o for the hydrolysis of **4–6** by [(Cu(II)tren)_{2.18}]_{0.89}PCD^{MeO} is illustrated in Figure 4. Kinetic data

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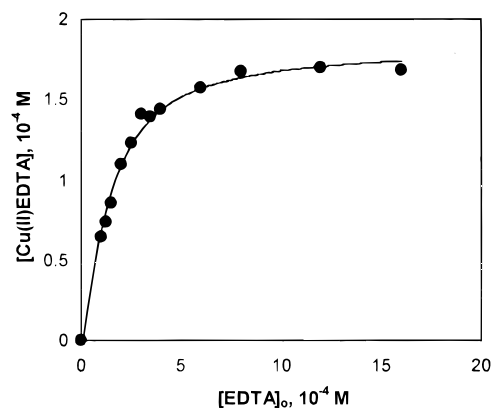


Figure 2. Plot of [Cu(II)EDTA] against [EDTA]₀ for equilibration between EDTA and [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO} at pH 8.00 and 25 °C.

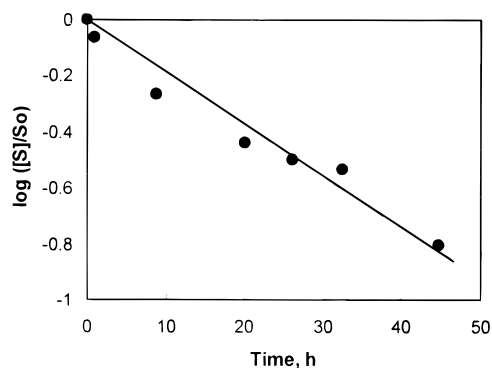


Figure 3. Plot of $\log [S]/S_0$ against time for the hydrolysis of **4** ($S_0 = 1.96 \times 10^{-4}$ M) catalyzed by [(Cu(II)tren)_{2.18}]_{0.89}PCD^{MeO} ($C_0 = 5.80 \times 10^{-4}$ M) at pH 8.00 and 50 °C. For each data point, concentration of glycine was measured from which $[S]$ was calculated. Analysis of the data led to $k_o = (4.24 \pm 0.50) \times 10^{-2}$ h⁻¹.

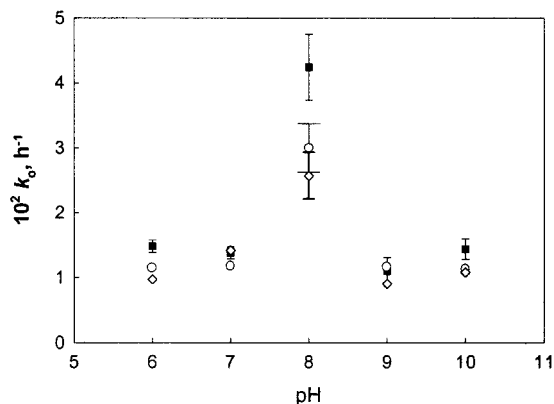


Figure 4. pH dependence of k_o for the hydrolysis of **4–6** ($S_0 = 1.96 \times 10^{-4}$ M: [■] **4**; (○) **5**; (◇) **6**) catalyzed by [(Cu(II)tren)_{2.18}]_{0.89}PCD^{MeO} ($C_0 = 5.80 \times 10^{-4}$ M) at 50 °C. Standard deviations of k_o are 6–18% of k_o values. Standard deviations are indicated as error bars for the data points of **4** at pH 6–10 and for those of **5** and **6** at pH 8.

collected for the hydrolysis of **4** catalyzed by [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO}, [(Cu(II)tren)_{1.43}]_{1.7}PCD^{MeO}, and [(Cu(II)tren)_{1.11}]_{0.73}PCD^{MeO} indicated that the optimum activity was also observed at pH 8. The values of k_o/C_0 for the hydrolysis of **4** catalyzed by the PCD-based catalysts at pH 8.00 are compared in Figure 5. The dependence of k_o on C_0 was examined for the hydrolysis **4** catalyzed by [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO} and the results are illustrated in Figure 6. Turnover kinetic behavior for the hydrolysis of **4** by [(Cu(II)tren)_{1.97}]_{0.74}PCD^{MeO} was examined under the conditions of $S_0 \gg C_0$ and the results are illustrated

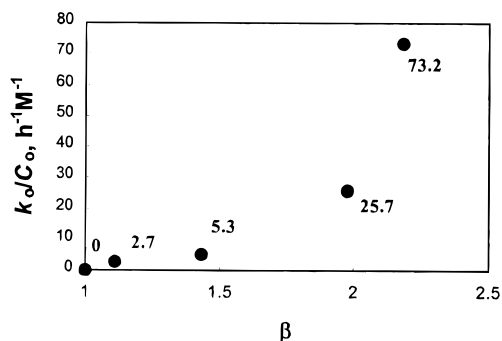


Figure 5. Values of k_o/C_o for various catalysts built with PCD with different β contents measured at pH 8.00 and 50 °C: $S_o = 1.96 \times 10^{-4}$ M; $C_o = 5.80 \times 10^{-4}$ M for $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$, 4.69×10^{-4} M for $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$, 1.11×10^{-3} M for $[(\text{Cu}(\text{II})\text{tren})_{1.43}]_{1.7}\text{PCD}^{\text{MeO}}$, and 4.74×10^{-4} M for $[(\text{Cu}(\text{II})\text{tren})_{1.11}]_{0.73}\text{PCD}^{\text{MeO}}$.

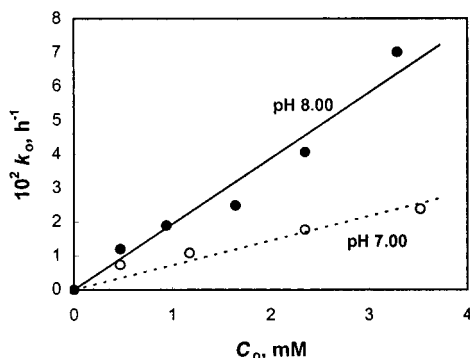


Figure 6. Dependence of k_o on C_o for the hydrolysis of **4** catalyzed by $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ at pH 7.00 (○) or 8.00 (●) and 50 °C: $S_o = 1.96 \times 10^{-4}$ M.

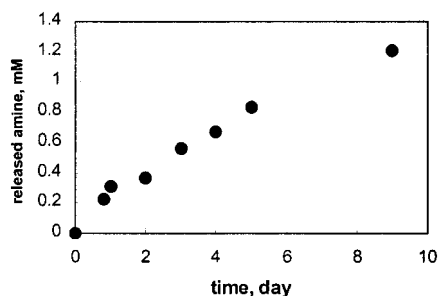


Figure 7. Release of glycine in the hydrolysis of **4** catalyzed by $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ at pH 8.00 and 50 °C in the presence of excess substrate: $S_o = 2.86 \times 10^{-3}$ M, $C_o = 4.22 \times 10^{-4}$ M.

in Figure 7. Due to the low C_o value, the reaction was slow. When Zn(II) or Ni(II) ion instead of Cu(II) ion was complexed to the tren moiety of $[(\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$, no catalytic activity in amide hydrolysis was observed. When **4** (1.96×10^{-4} M) was incubated with the Cu(II) complex of tren (1.09×10^{-3} M) at pH 6–10 and 50 °C for 2.5 days, hydrolysis of **4** was not observed as checked by HPLC for the release of cinnamate.

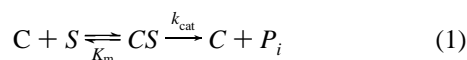
Discussion

Sites comprising one, two proximal, or three proximal Cu(II)-tren units are created on the backbone of cross-linked polystyrene. Only mononuclear sites are present in $[(\text{Cu}(\text{II})\text{tren})_1]_{1.7}\text{PCD}^{\text{MeO}}$, whereas a mixture of mononuclear, dinuclear, and trinuclear sites is obtained when MB is used as the precursor of the Cu(II)-tren units. Even when MB^{n-} with 3 or more anionic sites is attached to PCD, only one or two linkages can be formed between MB and PCD depending on the local geometry of PCD

in the vicinity of the site of the initial attachment. The highest content of Cu(II)-tren unit per MB site is obtained with $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$. For $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$, the fraction of MB sites containing three proximal Cu(II)-tren moieties is greater by 18% than that containing single Cu(II)-tren unit. The $\log K_f$ values for Cu(II)-tren moieties in $[(\text{Cu}(\text{II})\text{tren})_1]_{1.7}\text{PCD}^{\text{MeO}}$ and $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ measured at pH 8 indicate that the Cu(II) ions do not dissociate from the Cu(II)-tren units under the kinetic conditions. The value of $\log K_f$ for Cu(II)-tren increases upon attachment of Cu(II)-tren to PCD. This is attributable in part to suppressed protonation of tren in hydrophobic domains of PCD. Almost the same values of $\log K_f$ obtained for $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ and $[(\text{Cu}(\text{II})\text{tren})_1]_{1.7}\text{PCD}^{\text{MeO}}$ suggest that the dinuclear or trinuclear sites contain two or three discrete Cu(II)-tren units, respectively.

To exploit two or more proximal metal ions as catalytic or binding groups of artificial enzymes, it is desirable to arrange the proximal metal ions in convergent positions, pointing toward the complexed substrate. The three Cu(II)-tren units confined in MB take convergent positions, pointing toward the oxo bridge. When the three Cu(II)-tren units are connected to PCD, the rigidity of the polystyrene backbone may maintain the convergent geometry even after disassembly of the MB unit. The benzyl spacer that connects MB and the polystyrene backbone, however, should have some conformational freedom and allow flexibility in the relative conformations of the proximal Cu(II)-tren units. Conformational flexibility of the proximal Cu(II)-tren units would be advantageous in binding the substrate and relieving strains in the transition states for the chemical transformation of the bound substrate.

The catalytic efficiency of the proximal Cu(II)-tren units positioned on PCD is demonstrated with amide hydrolysis in the present study. For catalytic reactions proceeding through complex formation between the substrate and the artificial active sites built on synthetic polymers, the kinetic data are analyzed in terms of the Michaelis–Menten scheme (eq 1).^{11–14} Under the conditions of $C_o \approx [C] \gg [CS]$, pseudo-first-order kinetic behavior is predicted (eq 2). Since k_o is proportional to C_o for the reactions investigated in the present study (Figure 6), K_m is much greater than C_o . Even if C_o is not greater than S_o , the condition of $C_o \approx [C]$ is held when $K_m \gg C_o$ and $K_m \gg S_o$. When $K_m \gg C_o$, k_o is proportional to C_o and k_o/C_o corresponds to k_{cat}/K_m , representing the catalytic efficiency of the catalyst toward the substrate.



$$k_o = k_{\text{cat}}C_o/(C_o + K_m) \quad (2)$$

The linear dependence of k_o on C_o illustrated in Figure 6 reveals that K_m is much greater than 3 mM when C_o is expressed in terms of the MB site. If only trinuclear sites are catalytically active, however, the actual amount of the active site is considerably smaller than that of the MB sites. Then, the actual value of K_m is considerably smaller than the apparent value estimated on the basis of C_o expressed in terms of the amount of the MB sites. Since $k_{\text{cat}} \gg k_o$ under the conditions of $K_m \gg C_o$ (eq 2), k_{cat} is much greater than 0.02 h⁻¹ at pH 7.00 and then 0.07 h⁻¹ at pH 8.00 (Figure 6) for $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$.

The carboxy amides (**4**–**6**) are effectively hydrolyzed by the PCD-based catalysts synthesized with MB, whereas neutral amide **3** is not affected by the catalysts. Thus, the catalysts manifest selectivity toward carboxy amides. The carboxylate

anions of the substrate would be utilized in the complexation of the substrates to the active site. Recognition of carboxylate anion by one of the metal ions of the active site is, therefore, responsible for the substrate selectivity. The data summarized in Figure 4 reveal that $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$ does not considerably differentiate **4–6** which differ in the number of methylene units between the carboxy and the amide groups.

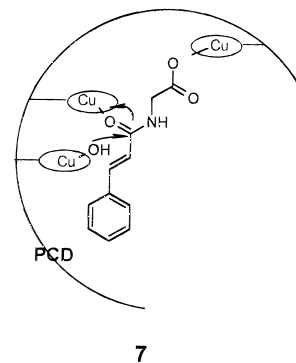
The catalytic activity is reduced remarkably as the average number (β) of $\text{Cu}(\text{II})\text{tren}$ per MB site is lowered (Figure 5). That $[(\text{Cu}(\text{II})\text{tren})_{1.17}]\text{PCD}^{\text{MeO}}$ has no catalytic activity reveals that the mononuclear sites are not active. A large difference is seen in catalytic activity between $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$ and $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$. The content of binuclear sites should not differ greatly for these two catalysts, excluding the possibility that the binuclear sites are the only catalytic sites. The trinuclear sites, therefore, should be catalytically active. For the catalyst with β value of 1.43, the content of the trinuclear site would be small and that of the binuclear site would be about 40% of the MB sites. Since k_o/C_o value for $[(\text{Cu}(\text{II})\text{tren})_{1.43}]_{1.7}\text{PCD}^{\text{MeO}}$ is much smaller than that for $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$, the catalytic activity of the binuclear site should be much smaller than the trinuclear site. Thus, even if the binuclear site is catalytically active, it should be much less active than the trinuclear site.³⁰

The results (Figure 7) of turnover kinetics confirm the catalytic nature of the action of the $\text{Cu}(\text{II})\text{tren}$ -containing PCD derivatives. In the experiment of Figure 7 carried out with 0.42 mM C_o (calculated on the basis of MB sites), 1.2 mM amine is produced in 9 days. If C_o is calculated in terms of the trinuclear sites since the amide is hydrolyzed mainly by the trinuclear sites, the number of the product formed per active site should be much larger than that (3) based on C_o calculated in terms of MB sites.

For Michaelis–Menten kinetics, k_{cat} represents the maximal rate constant achievable by the catalyst. When $C_o \gg K_m$, the substrate is completely complexed to the catalyst and k_o reaches the maximum value (k_{cat}). The k_{cat} value ($\gg 0.02 \text{ h}^{-1}$ at pH 7 and 50 °C; Figure 6) for the hydrolysis of **4** catalyzed by $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ may be compared with the k_o values (about $1 \times 10^{-6} \text{ h}^{-1}$ at pH 7 and 50 °C) reported for the spontaneous hydrolysis of small unactivated amides.^{31–33}

Metal ions acting as Lewis acid catalysts can play several catalytic roles in amide hydrolysis.^{34,35} For example, metal ions can bind the carbonyl oxygen of amide, promoting the reactivity of carbonyl group toward nucleophiles such as water molecule, hydroxide ion, or organic functional groups such as carboxylate ion. Metal-bound hydroxide anions can act as the nucleophiles that attack the carbonyl group of the amide substrate. Metal-bound water molecules can act as general acids to assist expulsion of leaving amines from the tetrahedral intermediates. Although it is not possible to assign exact catalytic roles to each of the three $\text{Cu}(\text{II})$ ions of the PCD-based catalysts investigated in the present study, the simplest mechanism that accounts for the involvement of three $\text{Cu}(\text{II})$ centers as well as the bell-shaped pH profile is indicated in 7. Here, the hydroxide ion bound to a $\text{Cu}(\text{II})$ center makes nucleophilic attack at the carbonyl group of the substrate activated by complexation to another $\text{Cu}(\text{II})$

center. The remaining $\text{Cu}(\text{II})$ center is used for recognition of the carboxylate anion of the substrate. To form the catalyst–substrate complex described in 7, one of the three $\text{Cu}(\text{II})$ ions of the active site should contain a hydroxo ligand and the other two $\text{Cu}(\text{II})$ ions contain aquo ligands in agreement with the bell-shaped pH profile of Figure 4. That the catalytic activity does not diminish rapidly as pH is raised or lowered from the optimal value suggests the complexity of ionization behavior of the $\text{Cu}(\text{II})$ -bound water molecules positioned in the trinuclear active site built on the PCD backbone.



Recently, several types of artificial enzymes with peptidase-like activities have been reported. Catalytic antibodies with reasonably high peptidase activities have been obtained,³⁶ although living organisms take crucial roles in the production of catalytic antibodies. Small metal complexes such as $\text{Pd}(\text{II})$ complexes³⁷ of analogues of ethylenediamine or $\text{Cu}(\text{II})$ complex³⁸ of [9]aneN₃ are the first fully synthetic catalysts reported to be capable of amide hydrolysis. Although the $\text{Pd}(\text{II})$ complexes manifested regioselectivity toward amide bonds adjacent to histidine or tryptophan, they were active only at acidic pH and sometimes in organic solvents.^{39,40} The highest catalytic activity (up to 10⁹-fold acceleration) reported so far for amide hydrolysis with artificial peptidases has been achieved with artificial metallopeptidases built with coordinatively polymerized synthetic bilayer membranes⁴¹ or $\text{Cu}(\text{II})$ complex of cyclen¹³ attached to a PCD derivative. These synthetic peptidases hydrolyzed proteins with half-lives of 1–20 min at 4 °C, but they failed to hydrolyze small amides.

Compared with synthetic peptidases reported previously,^{11,13,37–41} the catalysts prepared in the present study manifest much higher catalytic activity toward hydrolysis of small unactivated amides at neutral pHs. The k_{cat} value for $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ is estimated as $\gg 0.02 \text{ h}^{-1}$ at pH 7.00 and $\gg 0.07 \text{ h}^{-1}$ at pH 8.00 and 50 °C based on the kinetic data summarized in Figure 6. The activity of $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$ is about 3 times greater than that of $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.74}\text{PCD}^{\text{MeO}}$ (Figure 5). The activity of $[(\text{Cu}(\text{II})\text{tren})_{2.18}]_{0.89}\text{PCD}^{\text{MeO}}$ and $[(\text{Cu}(\text{II})\text{tren})_{1.97}]_{0.89}\text{PCD}^{\text{MeO}}$ may be compared with that ($k_{\text{cat}} = 0.18 \text{ h}^{-1}$ at pH 9, the optimum pH, and 25 °C) achieved in the hydrolysis of a carboxamide by the catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach.³⁶ This antibody is the catalytic

(30) Then, the numbers indicated in Figure 5 represent the relative contents of the trinuclear site in the MB sites. As mentioned above, the content of the trinuclear site is 18% or more when β is 2.18. Then, the content of the trinuclear site may be estimated as 1.3–3% and, consequently, that of the mononuclear site as 37–41% when β is 1.43.

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antibody with the highest peptidase activity reported to date. Considering the difference in pH and temperature, the catalytic activity of the PCD-based catalysts prepared in the present study appears to be comparable to or better than that of the catalytic antibody.

Another feature of natural peptidases reproduced by the PCD derivatives of the present study is the substrate selectivity. Carboxypeptidase A, for example, is an exopeptidase recognizing terminal carboxylate ions of proteins. Chymotrypsin, trypsin, and papain are some of endopeptidases that recognize side chains with unique structural features in the vicinity of the scissile amide bonds.¹ In this regard, the PCD derivatives with active sites comprising three proximal Cu(II)tren units manifest substrate selectivity toward amides containing proximal carboxylate ion.

Binuclear or trinuclear metal complexes can be prepared through stepwise synthetic pathways.^{42–45} For example, a trinuclear complex has been synthesized using calixarene as scaffold.⁴⁴ It would be very difficult, however, to prepare a three-dimensional molecular device with several convergent catalytic elements and to tune the geometry of the device by stepwise synthesis to obtain a highly effective artificial enzyme. In this regard, the novel strategy reported in the present study, transfer of metal-chelating sites confined in a prebuilt cage to a polymeric backbone, would be valuable in construction of active sites of artificial multinuclear metalloenzymes.

Experimental Section

Synthesis of Catalysts: PCD. This polymer was obtained by suspension copolymerization of chloromethylstyrene (7:3 mixture of *m* and *p* isomers) and divinylbenzene with benzoyl peroxide in the presence of cyclohexane and poly(vinyl alcohol) in water as described previously.¹³ The content of divinylbenzene was 2 mol % relative to chloromethylstyrene.

MB. Macrocyclic complex **1**(ClO₄)₄·2H₂O was synthesized as reported previously.²⁴

[MB]_αPCD. Attachment of MB to PCD to obtain [MB]_αPCD was carried out under nitrogen atmosphere with solvents degassed prior to use in synthesis. PCD (0.5 g; 3.3 residue mmol) and NaH (0.050 g; 2.1 mmol) were added to DMF (25 mL) taken from a fresh bottle and the resin was swollen for 1 day at room temperature. NaH (0.020 g, 0.83 mmol) was added to a DMF (5 mL) solution of MB (0.045 g; 4.0 × 10⁻⁵ mol) at room temperature and the mixture was stirred for 30 min and then the undissolved NaH powder was removed by filtration. Upon treatment with NaH, the color of the solution turned to brown from green. The brown solution of MB was added to PCD suspended in DMF, and the resulting mixture was shaken for 2 days at 50 °C. The speed of shaking employed in the synthesis of various derivatives of PCD in the present study was 60 rpm unless noted otherwise. When MB was attached to PCD, the resin became green. The green resin of [MB]_αPCD was collected by filtration and washed with 30 mL of methanol, 50 mL of water, and 50 mL of acetone. When it was attempted to vary the degree of cross-linkage of MB by PCD, *p*-nitrobenzoic acid (50, 100, or 200 mol % relative to MB: 0.13 g MB was treated with 0.030 g NaH for generation of MBⁿ⁻ when 100 or 200 mol % *p*-nitrobenzoic acid was used) was added to the solution of MBⁿ⁻ for partial protonation of the MBⁿ⁻.

[MB]_αPCD^{MeO}. The chloro groups of [MB]_αPCD were substituted with methoxide ion to obtain [MB]_αPCD^{MeO} by shaking [MB]_αPCD (prepared with 1.0 g PCD) with sodium methoxide (0.86 g, 16 mmol) in 100 mL of 1:1 (v/v) DMF-methanol at 50 °C for 30 h. The resin of

[MB]_αPCD^{MeO} was collected by filtration and washed with 30 mL of methanol and 30 mL of DMF. EPMA analysis indicated that the amount of unsubstituted chloromethyl group is negligible.

[(tren)_β]_αPCD^{MeO}. The MB moiety attach to the PCD backbone was disassembled by shaking [MB]_αPCD^{MeO} with 10 mL of concentrated HCl mixed with 10 mL of acetone at 25 °C for 2 days and by washing with 30 mL of acetone and 30 mL of methylene chloride. The color of the resin turned to yellow from green as MB moieties were disassembled. The amount of Cu(II) ion liberated during disassembly of MB with acid was quantified by ICP-AES. From the amount of Cu(II) ion, the content (α) of MB in [MB]_αPCD^{MeO} was calculated.

[(Cu(II)tren)_β]_αPCD^{MeO}. To [(tren)_β]_αPCD^{MeO} (1 g) stirred in a methylene chloride solution (10 mL) of triethylamine (2.9 g, 29 mmol) at room temperature for 3 h, 12 mL of DMF solution of CuCl₂ (3.3 mM) was added and the mixture was stirred at room temperature overnight. The resin of [(Cu(II)tren)_β]_αPCD^{MeO} was collected by filtration and washed thoroughly with water and an aqueous buffer solution of pH 6 (0.01 M 4-morpholineethanesulfonic acid (mes)). After [(Cu(II)tren)_β]_αPCD^{MeO} (0.040 g) was shaken in a mixture of 1 mL of concentrated HCl and 1 mL of acetone for 1 day at room temperature at the speed of 165 rpm, the resin was washed with 10 mL of water and the Cu(II) ion liberated was quantified by ICP-AES. From the amount of Cu(II) ion thus obtained and the value of α estimated as described above, the value of β was calculated.

***N,N*-Phthaloyl-*N*'-tert-butylloxycarbonyl-tren (Phth₂BOC-tren).** To a solution of tren (1.0 g, 6.8 mmol) and Na₂CO₃ (2.2 g, 20 mmol) in 100 mL of water and 20 mL of DMF, a DMF solution (5 mL) of *N*-carboxy phthalimide (3.0 g, 14 mmol) was added. After the solution was stirred at room temperature for 3 h, di-*t*-butyl carbonate (2.2 g, 10 mmol) was added to the mixture and the resulting solution was stirred for 2 h. Ethyl acetate (200 mL) was added to the mixture, and the resulting solution was washed with 0.1 M NaHCO₃ solution twice and with saturated NaCl solution. After drying with MgSO₄, the solvent was removed in vacuo. The product was recrystallized from ethyl acetate–hexane, mp 149.5–150 °C. ¹H NMR (300 MHz, CDCl₃) δ 1.35 (s, 9H), 2.84 (t, 2H), 2.88 (t, 4H), 3.12 (t, 2H), 3.72 (t, 4H), 5.00 (s, 1H), 7.27–7.77 (m, 8H). Anal. Calcd for C₂₇H₃₀N₄O₆: C, 63.44; H, 6.04; N 10.77. Found: C, 64.02; H, 5.97; N, 11.06.

[(Cu(II)tren)₁]_{1.7}PCD^{MeO}. To a solution of Phth₂BOC-tren (0.067 g, 0.13 mmol) in 20 mL of methylene chloride, trifluoroacetic acid (0.15 g; 1.3 mmol) was added, and the resulting mixture was stirred at room temperature for 30 min to remove the BOC group. The excess trifluoroacetic acid was removed by evaporation in vacuo. The resulting residue was dissolved in 50 mL of methylene chloride, to which PCD (1 g) and *N,N*-diisopropylethylamine (0.40 mL; 2.3 mmol) were added. The mixture was shaken at 25 °C for 5 days to attach the tren derivative to PCD. After the resin was washed with 100 mL of water and 100 mL of acetone, it was suspended in a 1:1 (v/v) mixture (200 mL) of DMF and methanol. After sodium methoxide (2.0 g; 37 mmol) was added to the mixture, the mixture was shaken at 50 °C for 2 days to replace chloro groups with methoxide. After the resin was washed with 50 mL of methanol and 50 mL of acetone, the mixture of the resin and methylamine (2.0 mL; 3.0 mmol) in 50 mL of methanol was shaken at 50 °C for 3 days to remove the phthalimide-protecting group, and the product ([[(tren)₁]_{1.7}PCD^{MeO}) was washed with 50 mL of methanol and 100 mL of acetone. Upon treatment of [[(tren)₁]_{1.7}PCD^{MeO} with DMF solution (6 mL) of 0.01 M CuCl₂, [(Cu(II)tren)₁]_{1.7}PCD^{MeO} was formed. Removal of the phthalimide-protecting group was confirmed by Cu(II) binding to the resulting tren unit. The amount of tren moieties attached to the resin was quantified by measuring the amount of Cu(II) ion released after treatment of [(Cu(II)tren)₁]_{1.7}PCD^{MeO} with 6 N HCl. EPMA analysis of [(Cu(II)tren)₁]_{1.7}PCD^{MeO} indicated the amount of unsubstituted chloromethyl group is negligible.

Synthesis of Substrates: *N*-Methyl Cinnamoyl Amide (3). This compound was prepared from cinnamoyl chloride and methylamine and was recrystallized from ethyl acetate–hexane, mp 111.5–112.5 °C (lit.⁴⁶ 110–111 °C).

***N*-Cinnamoyl Glycine (4), *N*-Cinnamoyl β-alanine (5), and *N*-Cinnamoyl γ-butyric Acid (6).** To a mixture of the corresponding

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amino acid (67 mmol) in 100 mL of MeOH at 4 °C, SOCl₂ (5 mL) was added, and the mixture was stirred for 30 min after the amino acid was dissolved. After evaporation of the solvent in vacuo, the residue (methyl ester) was dissolved in 100 mL of CHCl₃, which was extracted with 0.1 M NaHCO₃ solution (100 mL) twice and with saturated NaCl solution (100 mL), dried with MgSO₄, and then evaporated *in vacuo*. The solution of the residue and triethylamine (8.5 mL; 0.06 mol) were dissolved in 100 mL of CHCl₃, and cinnamoyl chloride (5.0 g; 30 mmol) dissolved in 10 mL of CHCl₃ was added to the solution at 4–25 °C. The mixture was stirred for 1 h further and then was washed with 100 mL of 0.1 N HCl, 100 mL of 0.1 N NaHCO₃, and 100 mL of saturated NaCl and dried with MgSO₄. The product (methyl ester of 4–6) was purified by silica column chromatography (eluted with 1:2 ethyl acetate–hexane). To the mixture of the methyl ester, 100 mL of water, and 20 mL of methanol, 1 g NaOH was added and the resulting mixture was stirred for 1 h at room temperature. After 100 mL of 0.1 N HCl was added, the mixture was extracted with 100 mL of ethyl acetate twice and the residue obtained after evaporation of ethyl acetate in vacuo was recrystallized from ethyl acetate–hexane, mp 194–195 °C (lit.⁴⁷ 193 °C) for 4, 145–146 °C for 5, and 84–85 °C for 6. For 5: ¹H NMR (300 MHz, CDCl₃) δ 2.47 (t, 2H), 3.43 (m, 2H), 6.620 (d, 1H), 7.19–7.43 (m, 7H); Anal. Calcd for C₁₂H₁₃NO₃: C, 65.66; H, 6.04; N, 6.19. Found: C, 65.74; H, 5.98; N, 6.39. For 6: ¹H NMR (300 MHz, CDCl₃) δ 1.76 (m, 2H), 2.27 (t, 2H), 3.27 (m, 2H), 6.60 (d, 1H), 7.25–7.48 (m, 7H); Anal. Calcd for C₁₃H₁₅NO₃: C, 66.94; H, 6.48; N, 6.00. Found: C, 66.93; H, 6.69; N, 5.86.

Measurements. Distilled and deionized water was used for preparation of buffer solutions. Stock solutions of sodium salts of 4–6 were prepared in water. In kinetic measurements, the stirring speed was controlled with a tachometer and temperature was controlled within ± 0.1 °C with a circulator. Prior to kinetic studies, the catalysts were swollen in buffer solutions for 3 h at 50 °C. Quantification of the amino

acids formed by amide hydrolysis was carried out by spectrofluorometric analysis using 2,3-naphthalenedicarboxaldehyde.²⁹ The amounts of the amino acids adsorbed onto the PCD resins were insignificant under the kinetic conditions. When the stirring speed of the reaction mixture was varied, *k*_o increased considerably as the stirring speed was raised to 800 rpm and reached the plateau value at 800–1000 rpm as checked with the hydrolysis of 4 catalyzed by [(Cu(II)tren)_{2.18}]_{0.89}PCD^{MeO} (*S*_o = 1.96 × 10⁻⁴ M, *C*_o = 5.80 × 10⁻⁴ M) at pH 8.00 and 50 °C. Kinetic data were, therefore, collected at the stirring speed of 1200 rpm. Stirring at 1200 rpm for more than 1 day caused partial breakage of the resins. It appears, however, that disintegration of the resin did not affect the kinetic data appreciably as shown by the data of Figure 3, presumably due to the small size of the substrate. To check the effect of ionic strength on the catalytic activity, kinetic data for the hydrolysis of 4 catalyzed by [(Cu(II)tren)_{2.18}]_{0.89}PCD^{MeO} were collected with 0.05, 0.1, or 0.2 M 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (hepes) (*S*_o = 1.96 × 10⁻⁴ M, *C*_o = 5.80 × 10⁻⁴ M) at pH 8.00 and 50 °C. Since *k*_o value was not affected by the changes in ionic strength, kinetic data were collected with 0.05 M buffer. Buffers (0.05 M) used for the kinetic studies were mes (pH 6), hepes (pH 7, 8), and boric acid (pH 9, 10). pH measurements were carried out with a Dongwoo Medical DP-880 pH/ion meter. UV–vis spectra were taken with a Cary 300 Bio UV/vis spectrophotometer. NMR spectra were recorded with a Bruker Avance DPX300 model. HPLC analysis was performed with a Waters 600 system. ICP-AES measurements were performed with a Shimadzu ICPS-1000IV model. EPMA analysis was performed with a CAMECA SX-57 model.

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