Artificial Peptidase with an Active Site Comprising a Cu(II) Center and a Proximal Guanidinium Ion. A Carboxypeptidase A Analogue

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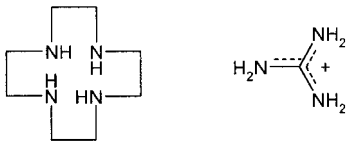
An immobile artificial metallopeptidase having a well-defined active site was constructed on the backbone of cross-linked polystyrene by adjoining a guanidinium moiety to the Cu(II) complex of a tetraaza ligand. The catalyst (CABP) and intermediate polymers were characterized by elemental analysis, IR, inductively coupled plasma measurement, electron probe microanalysis, test for primary amines, binding of Cu(II) ion, and complexation of *p*-nitrobenzoate ion. CABP effectively catalyzed amide hydrolysis of carboxyl-containing N-acyl amino acids. The catalytic rate of CABP in the hydrolysis of unactivated amides was comparable to that of the catalytic antibody with the highest peptidase activity reported to date. It is proposed that the guanidinium moiety of CABP recognizes the carboxylate anion of the substrate whereas the Cu(II) center participates in the cleavage of the amide bond of the complexed substrate. Several characteristic features of carboxypeptidase A were reproduced by CABP: catalytic action of the metal ion, participation of guanidinium in substrate recognition, hydrolysis of small unactivated amides, and substrate selectivity toward amide bonds adjacent to a carboxylate group.

Introduction

Molecular information both for substrate recognition and for thermodynamic efficiency of chemical transformation is needed for catalysis of biochemical reactions. Such information can be effectively carried by macromolecules,[1] and therefore, nature has developed enzymes by using polypeptides as the backbone. Macromolecules have also been employed as the skeletons of artificial enzymes such as catalytic antibodies[2−5] and catalytic polymers.[6−10] Synthetic polymers are useful as the backbone of effective and stable artificial enzymes in view of stability to heat and compatibility with organic solvents.

In the study of artificial enzymes constructed with macromolecules, major efforts are being made at present in the development of new strategies for designing active sites comprising catalytic and binding groups. For designing artificial active sites on the backbones of synthetic polymers, we have developed several new methods[10] such as random attachment of both binding and catalytic sites,[11] site-directed functionalization,[12] cross-linkage of catalytic elements with a macromolecular spacer,[13] transfer of catalytic elements confined in a cage,[14] and self-assembly from catalytic elements.[15] Here, we report construction of an active site of an artificial metallopeptidase by adjoining a binding site to a catalytic center attached to a macromolecular skeleton.

Amide linkages are stable to hydrolysis with a half-life of 500−1000 years at pH 7 and 25 °C.[16−18] Because of the difficulties in hydrolysis of unactivated amide bonds, nonenzymatic catalysis of amide hydrolysis has first been investigated with catalysts tethered to the substrate. Metal ions tethered to substrates manifested versatile catalytic repertories in amide hydrolysis.[19,20] Efforts to design peptidase-like catalysts separated from substrates, therefore, have been made mostly by employing metal ions as the catalytic centers.[11,14,21−26] For example, very high activity of protein hydrolysis (half-life of 10 min at pH 6 and 4 °C) was achieved when both the Cu(II) complex of cyclen (Cyc) and the guanidinium (Gua) group were



Cyc **Gua**

attached to a cross-linked polystyrene.[11] Here, Gua acted as the binding site that recognized the carboxylate ion of the protein substrate. The remarkable acceleration originated from 10^4-fold

(1) Dugas, H. *Bioorganic Chemistry*, 3rd ed.; Springer-Verlag: New York, 1996; pp 3, 172, and 255.
(2) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659−667.
(3) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391−395.
(4) Schultz, P. G.; Lerner, R. A. *Science* **1995**, *269*, 1835−1842.
(5) MacBeath, G.; Hilvert, D. *Chem. Biol.* **1996**, *3*, 433−445.
(6) Klotz, I. M. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; Chapter 2.
(7) Wulff, G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1812−1832.
(8) Suh, J. In *Polymeric Materials Encyclopedia*; Salamone, J. C., Ed.; CRC Press: Boca Raton, FL, 1996; pp 4210−4218 and 8230−8237.
(9) Hodge, P. *Chem. Soc. Rev.* **1997**, *26*, 417−424.
(10) Suh, J. *Adv. Supramol. Chem.* **2000**, *6*, 245−286.
(11) Jang, B.-B.; Lee, K. P.; Min, D. H.; Suh, J. *J. Am. Chem. Soc.* **1998**, *120*, 12008−12016.
(12) Suh, J.; Kwon, W. J. *Bioorg. Chem.* **1998**, *26*, 103−117.
(13) Suh, J.; Hah, S. S. *J. Am. Chem. Soc.* **1998**, *120*, 10088−10093.
(14) Moon, S. J.; Jeon, J. W.; Kim, H.; Suh, M. P.; Suh, J. *J. Am. Chem. Soc.* **2000**, *122*, 7742−7749.
(15) Suh, J.; Hong, S. H. *J. Am. Chem. Soc.* **1998**, *120*, 12545−12552.
(16) Bryant, R. A. R.; Hansen, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 5498−5499.
(17) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105−6109.
(18) Smith, R. M.; Hansen, D. A. *J. Am. Chem. Soc.* **1998**, *120*, 8910−8913.

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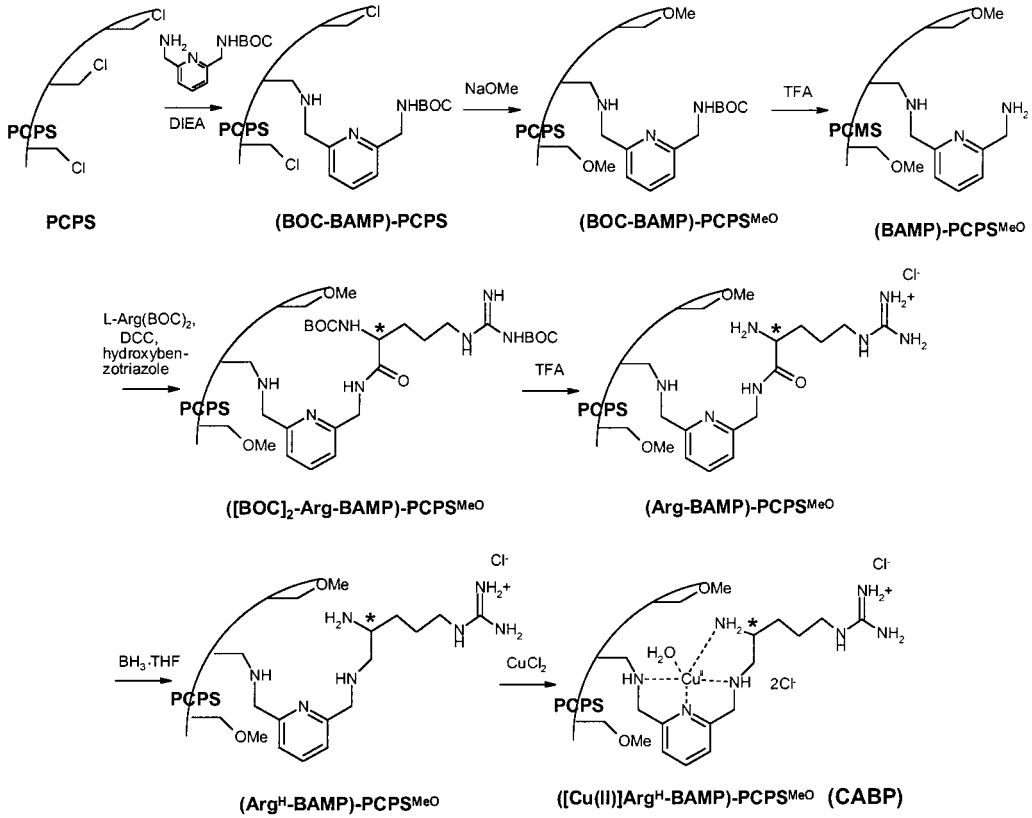
Scheme 1



enhancement in the catalytic activity of Cu(II)Cyc on attachment to the insoluble polymer. In addition, complexation between Gua and carboxylate, which is weak in water,[27] was facilitated in the microdomains of the resin. Thus, the cross-linked polystyrene provided a productive microenvironment both for complexation with substrate and for the catalytic conversion of the bound substrate.

Because Cu(II)Cyc and Gua were attached randomly, however, the active site was wide. It was estimated that about nine Cu(II)Cyc moieties and nine Gua moieties were present in the region covered by γ-globulin (MW 150 000).[11] Although the Cu(II)Cyc-containing resin was very effective in hydrolysis of γ-globulin, it failed to hydrolyze small peptides. In an effort to design an artificial peptidase that recognizes and hydrolyzes small amides, we improved the metal-containing polystyrene in the present study by attaching Gua in proximity to the metal center.

In the active site of carboxypeptidase A, a Zn(II) ion and Gua play essential catalytic roles.[20,28] Gua of carboxypeptidase A recognizes the carboxylate anion of the C-terminal amino acid residue of proteins thus making the enzyme an exopeptidase. The artificial enzyme prepared in the present study is designed as a mimic of carboxypeptidase A because both have active sites comprising the metal center and the proximal Gua ion. The metallopolymer of the present study indeed effectively hydrolyzes small unactivated amides, reproducing several characteristic features of carboxypeptidase A as described in this article.

Results

Synthesis and Characterization of the Catalyst. To design a well-defined active site on the polystyrene backbone, the Gua moiety is attached to a metal center anchored to partially chloromethylated cross-linked polystyrene (PCPS) by using arginine (Arg) as summarized in Scheme 1. In PCPS, which was purchased from a commercial source, 57% of the styryl moieties are chloromethylated and the degree of cross-linkage is 2%. The artificial active site constructed in ([Cu(II)]Arg^H-BAMP)-PCPS^{MeO} (CABP) contains the Cu(II) complex of a tetraaza ligand and an adjoining Gua group.

The PCPS derivatives prepared according to Scheme 1 were characterized as follows. For (BOC-BAMP)-PCPS, elemental analysis indicated that the content of 2,6-bis(aminomethyl)-pyridine (BAMP) was 0.70 mol % of the styryl moieties (0.052 mmol of BAMP per gram of resin). Electron probe microanalysis (EPMA) indicated that essentially all of the chloromethyl groups on the resin surface were substituted by methoxide in the preparation of (BOC-BAMP)-PCPS^{MeO}. In the synthesis of (BAMP)-PCPS^{MeO}, ([BOC]$_2$-Arg-BAMP)-PCPS^{MeO}, and (Arg-BAMP)-PCPS^{MeO}, appearance and disappearance of the primary amines were evidenced by the Kaiser test[29] using ninhydrin which forms Ruhemann's purple upon reaction with primary

(19) Suh, J. *Acc. Chem. Res.* **1992**, *25*, 273−279.
(20) Suh, J. *Perspect. Bioinorg. Chem.* **1996**, *3*, 115−149.
(21) Zhu, L. G.; Qin, L.; Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218−5224.
(22) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015−7016.
(23) Parac, T. N.; Ullmann, G. M.; Kostic, N. M. *J. Am. Chem. Soc.* **1999**, *121*, 3127−3135.
(24) Suh, J.; Oh, S. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1067−1070.
(25) Suh, J.; Moon, S. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2751−2756.
(26) Kumar, C. V.; Buranaprapuk, A.; Cho, A.; Chaudhari, A. *Chem. Commun.* **2000**, 597−598.
(27) Ariga, K.; Kunitake, T. *Acc. Chem. Res.* **1998**, *31*, 371−378.
(28) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62−69.
(29) Virender, K. S.; Stephen, B. H. K.; James, P. T.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.

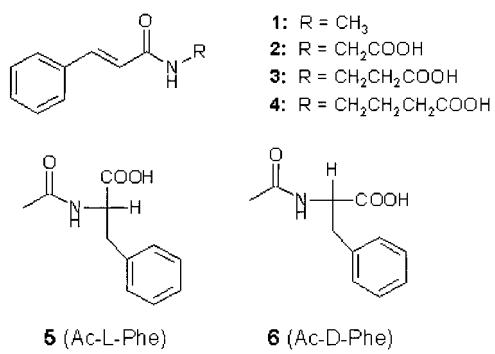
amines. The IR peak (1631 cm^{-1}) of the amide carbonyl group observed with ([BOC]$_2$-Arg-BAMP)-PCPS^{MeO} and (Arg-BAMP)-PCPS^{MeO} disappeared completely in (Arg^H-BAMP)-PCPS^{MeO}. EPMA revealed that the molar ratio of Cu to Cl on CABP surface was 1:3.5, in good agreement with the theoretical ratio of 1:3 (two Cl^- as counteranion of Cu(II) and one Cl^- as counteranion of Gua).

On addition of Cu(II) ion, (BAMP)-PCPS^{MeO} readily formed its Cu(II) complex, ([Cu(II)]BAMP)-PCPS^{MeO}. Metal-binding ability of the pyridyl moiety was lost in (Arg-BAMP)-PCPS^{MeO} but was restored when the amide group was reduced to obtain (Arg^H-BAMP)-PCPS^{MeO}. The contents of Cu(II) sites in ([Cu(II)]BAMP)-PCPS^{MeO} and CABP were 0.75 and 0.74 residue mol %, respectively, as determined by inductively coupled plasma (ICP) measurement of Cu(II) ion released on treatment of the resins with 6 N HCl. These contents are comparable to that (0.70 residue mol %) of BAMP in (BOC-BAMP)-PCPS as estimated by elemental analysis.

The formation constant (K_f) for the Cu(II) complex in ([Cu(II)]BAMP)-PCPS^{MeO} or CABP was measured by assuming[11] that binding of Cu(II) to a binding ligand is independent of succeeding bindings by analogy with the Langmuir isotherm. By the use of the method described previously,[11,30] ethylenediaminetetraacetate (EDTA) was used as the competing chelating reagent that extracts Cu(II) ion from the Cu(II)-binding sites built on the resin. The equilibrium constant (K_{ex}) for the Cu(II)-exchange reaction was estimated from nonlinear regression of the data for formation of Cu(II)EDTA by equilibration between EDTA and ([Cu(II)]BAMP)-PCPS^{MeO} or CABP (see the Supporting Information). When K_f was coupled with the formation constant for Cu(II)EDTA reported[31] in the literature, the average values of log K_f for the Cu(II) complexes were calculated as 15.90 ± 0.29 for ([Cu(II)]BAMP)-PCPS^{MeO} and 16.14 ± 0.20 for CABP at pH 9.00 and 25 °C. The K_f represents the apparent formation constant ([Cu(II)L]/[Cu(II)][L]$_t$: [L]$_t$ is the total concentration of the ligand existing in various ionization forms) and, thus, approaches the limiting value as pH is raised.

To obtain information on the content of the Gua portion, thermodynamic data for complexation of *p*-nitrobenzoate ion (NBA) to (Arg^H-BAMP)-PCPS^{MeO} were measured according to the procedure reported previously.[11] From the linear regression of the data (see the Supporting Information), the equilibrium constant (K_{com}) for the complexation was estimated as (6.5 ± 0.6) × 10^4. The regression also estimated the total amount of NBA that can be bound to the resin as 1.0 ± 0.1 residue mol % of the resin.

Kinetic Data for Amide Hydrolysis. The activity of CABP was examined with the hydrolysis of **1**−**6**. Neutral amide **1** was



5 (Ac-L-Phe) **6** (Ac-D-Phe)

not affected when stirred with CABP at pH 9.00 and 50 °C for 3 days. On the other hand, carboxyl-containing amides **2**−**6** were effectively hydrolyzed in the presence of CABP. Quantitative formation of cinnamic acid and phenylalanine (Phe) from the hydrolysis of **2**−**6** was confirmed by HPLC. Quantitative formation of amino acids from the hydrolysis of **2**−**6** including Phe was ensured by quantification with spectrofluorometric analysis using 2,3-naphthalenedicarboxaldehyde[32] as the coloring reagent. When Ni(II) or Zn(II) ion was complexed to (Arg^H-BAMP)-PCPS^{MeO} or no transition metal ion was added to (Arg^H-BAMP)-PCPS^{MeO}, no catalytic activity was observed in the amide hydrolysis.

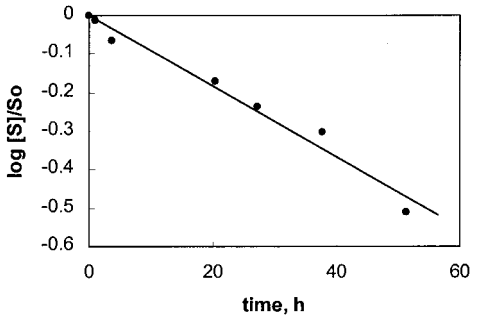


Figure 1. Plot of log [S]/S_0 against time for the hydrolysis of **5** (S_0 = 1.96 × 10^{-4} M) promoted by CABP (C_0 = 1.08 × 10^{-3} M) at pH 8.50 and 50 °C.

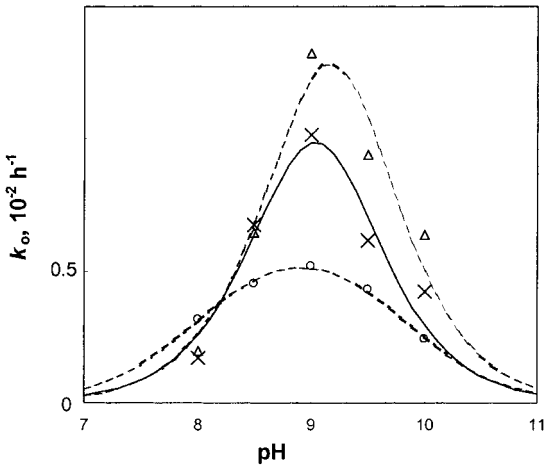


Figure 2. pH dependence of k_0 for hydrolysis of **2** (△), **3** (×), and **4** (○) (S_0 = 1.96 × 10^{-4} M) promoted by CABP (C_0 = 1.08 mM) at 50 °C.

A typical data set used for estimation of pseudo-first-order rate constants (k_0) is illustrated in Figure 1. Here, the concentration of Phe released from the hydrolysis of **5** was measured at various time intervals by using 2,3-naphthalenedicarboxaldehyde as the coloring reagent and was used to calculate the substrate concentration ([S]). The pH dependence of k_0 measured for the hydrolysis of **2**−**6** promoted by CABP is illustrated in Figures 2 and 3. For **2**−**4**, optimum activity was attained at pH 9 and the difference in k_0 among them was not large. For the hydrolysis of **2** promoted by CABP, k_0 was proportional to C_0 (Figure 4). For the hydrolysis of **5** and **6** promoted by CABP, k_0 was also proportional to C_0 (Figure 5), revealing small (<1.5) enantioselectivity toward **5**. Turnover kinetic behavior for the hydrolysis of **2** by CABP was examined under the conditions

(30) Lee, K.; Jang, B. B.; Kwon, W. J.; Suh, J. *J. Polym. Sci., Part A: Polym. Chem.* **1999**, *37*, 4117−4125.
(31) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum: New York, 1974; Vol. 1, p 204.
(32) de Mantigny, P.; Stobaugh, J. F.; Givens, R. S.; Carlson, R. G.; Srimivasachar, K.; Sternson, L. A.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1096−1101.

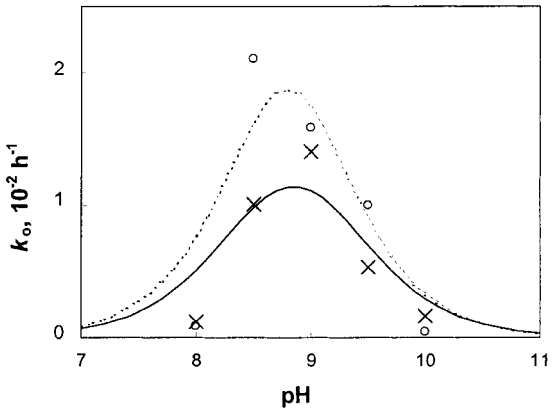


Figure 3. pH dependence of k_0 for hydrolysis of **5** (○) and **6** (×) ($S_0 = 1.96 \times 10^{-4}$ M) promoted by CABP ($C_0 = 1.08$ mM) at 50 °C.

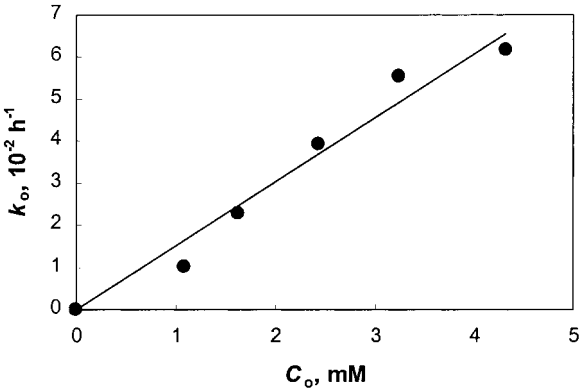


Figure 4. The dependence of k_0 on C_0 for the hydrolysis of **2** ($S_0 = 1.96 \times 10^{-4}$ M) promoted by CABP at pH 9.00 and 50 °C. Slope of the straight line is 15.2 ± 0.7 h^{-1} M^{-1}.

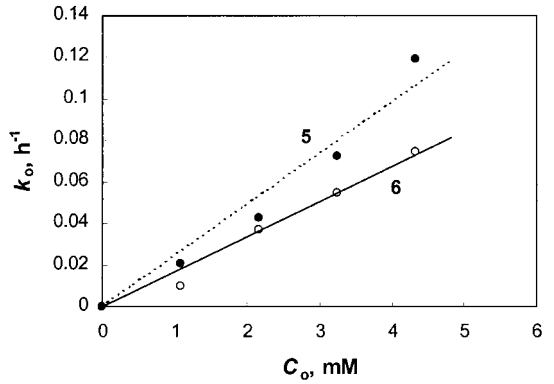


Figure 5. The dependence of k_0 on C_0 for the hydrolysis of **5** and **6** ($S_0 = 1.96 \times 10^{-4}$ M) promoted by CABP at pH 8.50 and 50 °C. Slopes of the straight lines are 24.7 ± 1.6 h^{-1} M^{-1} for **5** and 16.9 ± 0.7 h^{-1} M^{-1} for **6**.

of S_0 (2.86 mM) > C_0 (0.394 mM). Although the reaction was slow, release of 1.1 mM glycine (Gly) was observed in 22 days (Figure 6).

Discussion

The active site of an artificial metallopeptidase is constructed on the backbone of cross-linked polystyrene by adjoining Gua to a Cu(II) complex of a tetraaza ligand in this study. The active site is built on the insoluble polymer instead of synthesizing a small soluble compound for several reasons: We were interested in developing novel methodologies for designing artificial enzymes on the backbone of synthetic polymers. Insoluble polymer provides additional advantages in practical applications

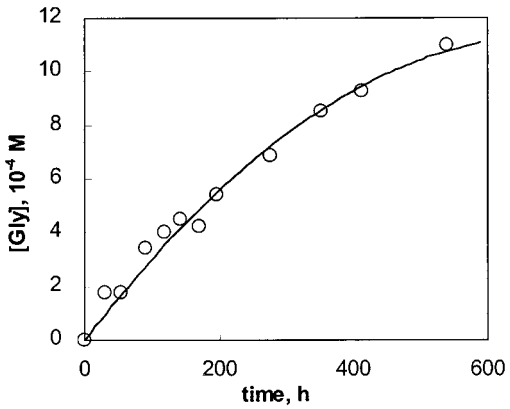


Figure 6. Concentration of Gly released from the hydrolysis of **2** by CABP under the condition of S_0 (2.86 mM) > C_0 (0.394 mM) at pH 9.00 and 50 °C. No reaction occurred in the absence of CABP.

such as continuous processing and easy separation of catalysts. Most of all, this study was inspired by the 10^4-fold increase[11] in intrinsic proteolytic activity of Cu(II)Cyc upon attachment to the backbone of a cross-linked polystyrene. In addition, the ability of Gua to recognize carboxylate ion should be improved in the hydrophobic microdomains of polystyrene.

CABP was synthesized through the path summarized in Scheme 1. The intermediate polymers included in Scheme 1 as well as CABP were characterized by various methods such as elemental analysis, IR, ICP, EPMA, test for primary amines, binding of Cu(II) ion, and complexation of *p*-nitrobenzoate ion.

The log K_f values for the Cu(II) complexation in ([Cu(II)]-BAMP)-PCPS^{MeO} (15.90 ± 0.29) and CABP (16.14 ± 0.20) do not differ appreciably although one more nitrogen is involved as the ligating atom in CABP. Comparison of the two log K_f values is, however, complicated by a difference in the degree of protonation between the two ligand systems. The log K_f values ensure that the Cu(II) ion is totally bound to the tetraaza ligand of CABP under the conditions of kinetic measurements.

The total amount of NBA that can be bound to (Arg^H-BAMP)-PCPS^{MeO} would correspond to the content of Gua in the resin if NBA forms a 1:1-type complex with Gua and if NBA does not interact with any other part of the resin. The content of Gua in the resin would be the same as that of the Cu(II) binding site if the synthetic steps of Scheme 1 proceeded quantitatively. The amount (1.0 ± 0.1 residue mol %) of NBA that can be bound to the resin is somewhat larger than the content (0.74 residue mol %) of the Cu(II)-binding site. This may be ascribed to the formation of Gua$(NBA)_2$ by some of the Gua moieties of the resin by analogy with the complex formation[33] between one Gua and two phosphate ions.[34]

CABP effectively promotes hydrolysis of **2**−**6** under the conditions of $C_0 \gg S_0$. That neutral amide **1** is not affected by CABP indicates that carboxylate groups of **2**−**6** play an essential role in the catalytic mechanism, most probably through complexation with Gua moiety of CABP. For reactions of various catalysts built on a polystyrene backbone, kinetic data have been analyzed in terms of the Michaelis−Menten scheme (eq 1)

(33) Adams, J. M.; Small, R. W. H. *Acta Crystallogr., Sect. B* **1976**, *32*, 832−835.

(34) Although NBA is complexed to the resin with a high K_{com} value, complexation of aliphatic carboxylates to the resin is much weaker as shown by the weak complexation of **2**−**6** to CABP ($K_m \gg 4.3$ mM; Figures 4 and 5). In addition, complexation of phenylacetate (1×10^{-4} M) to Gua-containing polystyrene resins was negligible. Strong complexation of carboxylate anions to Gua-containing polystyrenes was observed only when the carboxylate group was directly attached to aromatic rings.

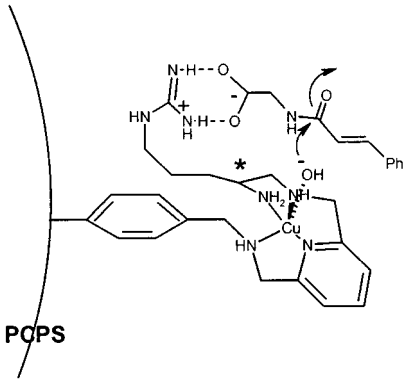
which involves complexation of the substrate with the catalyst.[11−15] Under the conditions of $C_0 \approx [C] \gg [CS]$, pseudo-first-order kinetic behavior is predicted (eq 2). When $K_m \gg C_0$, eq 2 predicts that k_0 is proportional to C_0, without showing the saturation kinetic behavior. Because k_0 is proportional to C_0, K_m is much larger than C_0 for the reactions investigated in the present study: K_m for the CABP-promoted hydrolysis of **2**−**6** is much greater than the highest C_0 value (4.3 mM). Under the conditions of $K_m \gg C_0$, $k_0 \ll k_{cat}$; k_{cat} is much greater than the highest k_0 value observed in the present study.

$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} C + P_i \quad (1)$$

$$k_0 = k_{cat}C_0/(C_0 + K_m) \quad (2)$$

For Michaelis−Menten kinetics, k_{cat} represents the maximal rate constant achievable when the substrate is completely bound to the active site in the presence of excess catalyst ($C_0 \gg K_m$). The k_{cat} value ($\gg 0.06$ h^{-1} at pH 9.00 and 50 °C; Figure 4) for the hydrolysis of **2** and that ($\gg 0.12$ h^{-1} at pH 8.50 and 50 °C; Figure 5) for the hydrolysis of **5** promoted by CABP may be compared with k_0 (about 2×10^{-6} h^{-1} at pH 9 and 50 °C)[16−18] for the spontaneous hydrolysis of small unactivated amides. The k_{cat} values may also be compared with k_{cat} (0.18 h^{-1} at pH 9, the optimum pH, and 25 °C)[35] achieved in amide hydrolysis by the catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach. This is the catalytic antibody with the highest peptidase activity reported to date.

The mechanism for the catalysis by CABP is suggested as **7** or **8** in view of catalytic roles[19,20] discovered for metal ions



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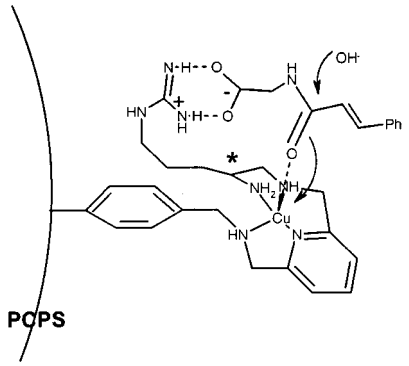

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acting as Lewis acid catalysts in amide hydrolysis. As mentioned above, interaction between Gua of CABP and carboxylate group of **2**−**6** is essential to the catalysis. The conformation of the active site appears to be flexible enough to accommodate **2**−**4**, leading to the similar catalytic activity (Figure 2) manifested toward **2**−**4**. Enantioselectivity manifested by CABP toward **5** and **6** (Figures 3 and 5) is not large although the active site is chiral because of attachment of L-arginine to BAMP. The chiral centers of the catalyst and the bound amide substrate are not positioned in close proximity, resulting in the small enantioselectivity.

Although many studies were carried out for peptide hydrolysis promoted by metal ions tethered to peptides,[19,20] true catalysts for peptide hydrolysis have been reported only recently. Synthetic or semisynthetic catalysts for peptide hydrolysis include catalytic antibodies,[35] small metal complexes such as Pd(II) complexes[21,23] of analogues of ethylenediamine or Cu(II) complex[22] of [9]aneN$_3$, and synthetic polymers[11,13,14,24,25] with artificial active sites. Besides the catalytic antibodies, catalytic hydrolysis of small amides at near neutral pH has been achieved only by CABP prepared in the present study and the artificial metallopeptidase with trinuclear active sites prepared recently[14] in this laboratory. The artificial metallopeptidase with trinuclear active sites has been constructed on a polystyrene backbone with a trinuclear site confined in a molecular bowl. The three metal ions of the trinuclear active site were utilized in complexation of the carboxylate group of the substrate and catalytic conversion of the complexed amide. On the other hand, CABP utilizes Gua in complexation of the carboxylate of the substrate and the Cu(II) center in cleavage of the amide bond of the complexed substrate by close analogy with carboxypeptidase A.

In carboxypeptidase A,[20,28] the active-site Zn(II) ion plays an essential catalytic role in cleavage of the amide bond of the complexed substrate whereas Gua of Arg-145 recognizes the carboxylate anion of the substrate. Important features of carboxypeptidase A reproduced by CABP include essential catalytic action of the metal ion, participation of Gua in substrate recognition, hydrolysis of unactivated amides,[36] and substrate selectivity toward amide bonds adjacent to a carboxylate group. Carboxypeptidase A utilizes additional catalytic groups such as Glu-270 and Tyr-248. A better synthetic analogue of carboxypeptidase A would be obtained by introducing additional catalytic groups such as carboxyl and/or phenol groups in the vicinity of the active site comprising the metal center and Gua.

Experimental Section

***N-t*-(BOC)-2,6-bis(aminomethyl)pyridine.** To a solution of BAMP[37] (1.4 g, 10 mmol) in 100 mL of CH_2Cl_2, a solution of 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (2.4 g, 9.7 mmol) in 10 mL of CH_2Cl_2 was added dropwise over a period of 1 h at room temperature, and the mixture was stirred for 3 h. The resulting solution was extracted with 100 mL of 0.1 M $NaHCO_3$ three times, dried with $MgSO_4$, and evaporated in vacuo. The residue was purified with SiO_2 column chromatography (1:3:0.01 ethyl acetate−hexane−30% ammonia solution). The fraction with $R_f = 0.5$ was collected to obtain oily material of *N-t*-(BOC)-2,6-bis(aminomethyl)pyridine. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45 (s, 9H), 3.28 (s, 2H), 3.94 (s, 2H), 4.38 (s, 2H), 6.05 (s, 1H), 7.15 (d, 2H), 7.60 (t, 1H). Anal. Calcd for $C_{12}H_{19}N_3O_2$: C, 60.74; H, 8.07; N, 17.71. Found: C, 60.65; H, 8.20; N, 17.63.

(BOC-BAMP)-PCPS. PCPS was purchased from Fluka. To a suspension of PCPS (10 g, 76 residue mmol of styrene monomer) in

(35) Gao, C.; Lavey, B. J.; Lo, C.-H. L.; Datta, A.; Wentworth, P., Jr.; Janda, K. D. *J. Am. Chem. Soc.* **1998**, *120*, 2211−2217.

(36) On the basis of the rate data reported in the literature (Coleman, J. E.; Valllee, B. L. *Biochemistry* **1962**, *1*, 1083−1092. Kaiser, B. L.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 36−41.), k_{cat}/K_m for CPA-catalyzed hydrolysis of **5** at pH 7 and 25 °C is estimated as about 30 s^{-1} M^{-1}. On the other hand, k_2 for hydrolysis of **5** promoted by CABP is 24.7 h^{-1} M^{-1} (Figure 5) at pH 8.5 and 50 °C.

(37) Lions, F.; Martin, K. V. *J. Am. Chem. Soc.* **1957**, *79*, 2733−2738.

200 mL of tetrahydrofuran (THF), *N*-*t*-(BOC)-2,6-bis(aminomethyl)-pyridine (0.20 g, 0.84 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (0.15 mL, 0.85 mmol) were added, and the mixture was shaken at 45 rpm and 50 °C for 3 days. In this study, solvents used for modification of PCPS derivatives were degassed for 30 min. The product resin was collected by filtration, washed with 500 mL of acetone and 500 mL of water, and dried in vacuo. Elemental analysis indicated that the content of BAMP in the resin was 0.70 mol % of the styryl moieties (0.052 mmol of BAMP per gram of resin).

(BOC-BAMP)-PCPS^{MeO}. A suspension of (BOC-BAMP)-PCPS (8 g, 0.42 residue mmol of BAMP) in 100 mL of *N*,*N*-dimethylformamide (DMF) containing sodium methoxide (50 mL, 28% methanol solution, 260 mmol) was shaken at 45 rpm and 50 °C for 3 days. The product resin was collected by filtration, washed with 500 mL of acetone and 500 mL of water, and dried in vacuo. EPMA results indicated that the amount of unreacted chloromethyl groups on the resin surface was less than 2% of the original amount.

(BAMP)-PCPS^{MeO}. (BOC-BAMP)-PCPS^{MeO} (5 g, 0.26 residue mmol BAMP) was shaken in the mixture of 20 mL of trifluoroacetic acid and 50 mL of CH_2Cl_2 at 150 rpm and 25 °C for 2 h. The product resin was collected by filtration, washed with 100 mL of 1 N HCl, 100 mL of CH_2Cl_2 containing 5% DIEA, 500 mL of acetone, and 500 mL of water, and dried in vacuo. Formation of primary amines was confirmed by the Kaiser test.[29] Quantification of the primary amines in the resins prepared in the present study by the Kaiser test was not, however, possible because of adsorption of Ruhemann's purple onto the resins.

([BOC]$_2$-Arg-BAMP)-PCPS^{MeO}. A mixture of *N*,*N*′-di-*t*-(BOC)-L-arginine[38] (0.9 g, 2.4 mmol), *N*,*N*′-dicyclohexylcarbodiimide (0.5 g, 2.4 mmol), 1-hydroxybenzotriazole hydrate (0.33 g, 2.4 mmol), and (BAMP)-PCPS^{MeO} (3 g, 0.16 residue mmol BMBP) in 100 mL of DMF was shaken at 45 rpm and 50 °C for 3 days. The product resin was collected by filtration, washed with 500 mL of acetone and 500 mL of water, and dried in vacuo. Disappearance of primary amines was confirmed by the Kaiser test. FT-IR: 1631 cm^{-1} (C═O).

(Arg-BAMP)-PCPS^{MeO}. A mixture of ([BOC]$_2$-Arg-BAMP)-PCPS-^{MeO} (3 g, 0.16 residue mmol BAMP) and trifluoroacetic acid (20 mL) in 50 mL of CH_2Cl_2 was stirred at room temperature for 2 h. The product resin was collected by filtration, washed with 100 mL of 1 N HCl, 100 mL of CH_2Cl_2 containing 4% DIEA, 500 mL of acetone, and 500 mL of water, and dried in vacuo. Formation of the primary amine was confirmed by the Kaiser test. FT-IR: 1631 cm^{-1} (C═O).

(Arg^H-BAMP)-PCPS^{MeO}. To a suspension of (Arg-BAMP)-PCPS-^{MeO} (2.5 g, 0.13 residue mmol BAMP) in 50 mL of THF, 10 mL of a THF solution of 1 M BH_3·THF (purchased from Aldrich) was added, and the resulting mixture was gently stirred at room temperature overnight.[39] The product resin was collected by filtration, washed with 100 mL of 1 N HCl, 100 mL of CH_2Cl_2 containing 4% DIEA, 500 mL of acetone, and 500 mL of water, and dried in vacuo. The FT-IR peak at 1631 cm^{-1} disappeared.

([Cu(II)]Arg^H-BAMP)-PCPS^{MeO}. In a 0.1 M $CuCl_2$·$2H_2O$ solution in DMF (20 mL), (Arg^H-BAMP)-PCPS^{MeO} (2.5 g, 0.13 residue mmol of BAMP) was suspended, and the resulting mixture was shaken at 150 rpm and 25 °C for 1 day. The product resin was collected by filtration, washed with 200 mL of DMF, 500 mL of water, and 100 mL of pH 5.5 buffer. Then, the resin was shaken in 50 mL of pH 8.0 buffer at 150 rpm and 25 °C for 1 day, washed with 500 mL of water and 200 mL of acetone, and dried in vacuo. EPMA analysis indicated that the molar ratio of Cu to Cl on the resin surface was 1:3.5.

Substrates. Amides **1**−**5** were prepared as reported previously,[14] whereas **6** and **7** were purchased from Sigma.

Measurements. Distilled and deionized water was used for preparation of buffer solutions. Stock solutions of sodium salts of **2**−**7** 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, CABP was swollen in buffer solutions for 3 h at 50 °C. Adsorption onto CABP of the amino acids formed from **2**−**6** was insignificant under the kinetic conditions. Catalyst concentration (C_0) was taken as the concentration of the Cu(II)-containing active site obtainable when it is assumed that the resin is dissolved in the buffer solution. ICP data were used in calculation of the Cu(II) content of CABP. When the stirring speed of the reaction mixture was varied, k_0 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 **2** promoted by CABP ($S_0 = 1.96 \times 10^{-4}$ M, $C_0 = 1.08 \times 10^{-3}$ M) at pH 9.00 and 50 °C. Kinetic data were, therefore, collected at the stirring speed of 1200 rpm. To check the effect of ionic strength on the catalytic activity, kinetic data for the hydrolysis of **2** promoted by CABP were collected with 0.05, 0.1, or 0.2 M boric acid ($S_0 = 1.96 \times 10^{-4}$ M, $C_0 = 1.08 \times 10^{-3}$ M) at pH 9.00 and 50 °C. Because the k_0 value decreased somewhat as buffer concentration was raised, kinetic data were collected with 0.05 M buffer without any added salt. Buffers (0.05 M) used for the kinetic studies were 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (pH 8) and boric acid (pH 8.5−10). Quantification of hydrolysis products was performed after the substrate was incubated with the catalyst for at least 10 half-lives. Measurements of pH were carried out with a Dongwoo Medical DP-880 pH/ion meter. Spectrofluorometric measurement was carried out with a JASCO FP-750 model. IR spectra were recorded with a Perkin-Elmer FT2000 spectrophotometer. HPLC analysis was performed with a Waters 600 system. Inductively coupled plasma-atomic emission spectrometry (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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Supporting Information Available: Plot of [Cu(II)EDTA] against $[EDTA]_0$ for the Cu(II) transfer from CABP to EDTA at pH 9.00 and 25 °C and plot of the concentration ($[NBA]_b$) of NBA bound to (Arg^H-BAMP)-PCPS^{MeO} against the concentration ($[NBA]_u$) of NBA uncomplexed to the resin (Figures S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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(38) Schnabel, E. *Liebigs Ann. Chem.* **1967**, *702*, 188−196.
(39) Brandes, S.; Denat, F.; Lacour, S.; Rabiet, F.; Pullumbi, P.; Guilard, R. *Eur. J. Org. Chem.* **1998**, 2349−2360.