Immobile Artificial Metalloproteinase Containing Both Catalytic and Binding Groups

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Abstract: Poly(chloromethylstyrene-*co*-divinylbenzene) (PCD) with 2% cross-linkage is developed as a backbone of immobile artificial enzymes. As the first artificial enzyme built on PCD, an artificial metalloproteinase is prepared by attaching the Cu(II) complex of cyclen as the catalytic center and guanidinium ion as the binding unit. The PCD derivatives prepared were characterized by scanning electron microscopy, IR spectroscopy, elemental analysis, titration of chloromethylphenyl moiety with triethylamine, complexation of *p*-nitrobenzoate ion to the guanidinium moiety, quantification of the cyclen moieties retaining high affinity for the Cu(II) ion, and determination of log K_f for the Cu(II) binding sites. The proteinase activity was measured with γ-globulin (Gbn) by following cleavage of the two chains of Gbn by electrophoresis. The catalytic activity of the Cu(II) complex of cyclen toward Gbn was enhanced by more than 10^4 times upon attachment to PCD. Gbn complexed to the PCD derivative containing both Cu(II)-cyclen and guanidinium moieties was cleaved by hydrolysis into many pieces with a half-life as short as 10−30 min at pH 4.5−7 and 4 °C. Kinetic data revealed that guanidinium ions attached to PCD acted as effective binding sites for the protein, contributing considerably to the overall catalytic power of the immobile artificial metalloproteinase.

Introduction

The design of biomimetic catalysts is intensively studied in various branches of chemistry and biology.[1−5] Thus, artificial enzymes have been prepared by using organic host molecules,[1] mononuclear or multinuclear coordination compounds,[2] proteins,[3,4] clusters of amphiphiles such as micelles or vesicles,[5] and synthetic polymers.[6] The major aim in the area of artificial enzymes is to design artificial systems that reproduce characteristics of enzymatic action such as complex formation with substrates, large rate acceleration, and high selectivity.

As nature chose polypeptide as the backbone of the active sites, macromolecules have been employed as the skeletons of artificial enzymes such as catalytic antibodies[3] and synzymes[6] (synthetic polymers with enzyme-like activities[6a]). When macromolecules are used as the skeletons of artificial enzymes, it is difficult to obtain information on the structure and mechanism of the catalyst. At present in the area of artificial enzymes such as catalytic antibodies or synzymes, 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 fully characterized and the mechanism of catalysis may not be understood on the molecular level.

For practical applications, enzymes are frequently immobilized.[7] In this regard, development of methodologies for designing immobile artificial enzymes is an important subject in the area of biomimetic catalysts. Reactants supported on insoluble polymers are widely used in synthetic reactions such as combinatorial syntheses. In addition, many synthetic catalysts have been attached to insoluble polymer supports. Previous studies on organic reactions supported on insoluble polymers revealed several unique features of the reactions taking place in the gel phases on swollen beads.[6f] However, designing active sites of artificial enzymes on insoluble polymers has not been systematically investigated.

The most significant technique developed so far for designing active sites of insoluble artificial enzymes is molecular imprinting. In molecular imprinting, monomers with functional groups are bound by a template and then copolymerized under conditions leading to the formation of highly cross-linked polymers with chains in fixed arrangements.[6e] After removal

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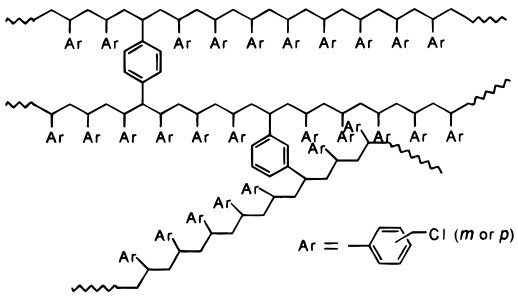
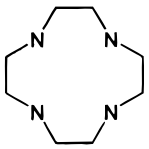
of the template, catalytic sites can be generated. A large portion of the catalytic sites prepared by molecular imprinting would be embedded within the polymeric body and, therefore, substrates may have limited access to those sites. Morphology of the polymeric materials obtained by molecular imprinting is not readily controlled.

To overcome these limitations, we attempted to synthesize artificial enzymes by modification of the surface of porous polymeric beads with sufficient mechanical strengths. For this purpose, poly(chloromethylstyrene-*co*-divinylbenzene) (PCD) was chosen, since preparation and characterization of PCD have been reported.[8] 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. Moreover, the microenvironment of the polymer surface can be adjusted by modification of the chloromethylphenyl groups.

In the present study, the Cu(II)-complex of 1,4,7,10-tetraazacyclododecane (cyclen) is attached to PCD as a catalytic group in view of versatile catalytic repertories[9] of metal ions in organic reactions. In addition, the guanidinium group is attached to PCD in view of its ability to recognize anions such as carboxylate and phosphate ester anions.[10,11] One of the major problems faced in designing artificial enzymes on the skeletons of synthetic polymers has been the lack of specific binding sites. The guanidinium ions employed in the present study can overcome this problem. In this paper, we report the first immobile artificial proteinase, together with 10^8-fold acceleration in the hydrolysis of γ-globulin (Gbn) by the immobile artificial proteinase equipped with both catalytic and binding sites.



PCD



cyclen

Experimental Section

PCD. This polymer was obtained by suspension copolymerization of chloromethylstyrene (10.9 g, 0.0714 mol; 7:3 mixture of *m* and *p* isomers purchased from Aldrich) and divinylbenzene (net weight 0.186−0.929 g, 1.43−7.14 mmol; mixture of isomers purchased from Aldrich) with benzoyl peroxide (0.83 g) in the presence of cyclohexane (12 mL) and poly(vinyl alcohol) (0.080 g) in 350 mL of water at 80 °C for 10 h according to the procedure described in the literature.[8] The resins with dry bead sizes of 100−300 μm were collected with sieves. In the PCD used for the modification described below, the content of divinylbenzene was 2 mol % relative to chloromethylstyrene. IR: 3020 (aromatic C−H stretch), 2850−2950 (aliphatic C−H stretch), 1264 cm^{-1} (C−Cl bend).

[Gua]$_3^{Cl}$PCD. Agmatine sulfate ((4-aminobutyl)guanidine sulfate) was converted to agmatine chloride by treatment with Amberlite IRA-400(C1) anion exchange resin. Agmatine chloride (0.35 g, 2.1 mmol) was dissolved in 200 mL of dimethyl sulfoxide (DMSO) and PCD (4 g) was suspended in the resulting solution. After the mixture was shaken at 20 rpm for 3 days at 80 °C, the modified resin ([Gua]$_3^{Cl}$PCD) was collected by filtration, washed with DMSO, water, and acetone, and then dried *in vacuo* for 1 day. IR: 1646 and 1512 (N−H bend), 1160 cm^{-1} (C−N stretch).

[Gua]$_3^{MeO}$PCD. [Gua]$_3^{Cl}$PCD (0.1g) was suspended in a methanolic solution (2.5 mL) of 5 M sodium methoxide and the mixture was shaken at 20 rpm for 1 day at 60 °C. The resulting resin ([Gua]$_3^{MeO}$PCD) was washed with methanol, water, and acetone and then dried for 1 day *in vacuo*. IR: 1089 cm^{-1} (C−O stretch).

[Cyc]$_3$[Gua]$_3^{Cl}$PCD. The residue obtained by lyophilization of a solution of cyclen tetrahydrochloride salt (1.0 g, 3.1 mmol) and NaOH (0.50 g, 13 mmol) dissolved in 20 mL water was extracted twice with 25 mL of chloroform. After evaporation of chloroform *in vacuo*, cyclen was recovered. [Gua]$_3^{Cl}$PCD (4 g) was suspended in a solution of cyclen (0.36 g, 2.1 mmol) dissolved in 100 mL of dimethyl formamide and 100 mL of chloroform and the resulting mixture was shaken at 20 rpm for 2 days at 60 °C. The resulting resin ([Cyc]$_3$[Gua]$_3^{Cl}$PCD) was washed with chloroform, water, and acetone, and then dried for 1 day *in vacuo*. IR: 1646 and 1510 (N−H bend), 1160−1100 cm^{-1} (C−N stretch).

[Cyc]$_3$[Gua]$_3^{MeO}$PCD. This resin was prepared by treating [Cyc]$_3$[Gua]$_3^{Cl}$PCD with sodium methoxide as described above for [Gua]$_3^{MeO}$PCD. IR: 1089 cm^{-1} (C−O stretch).

[Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD. [Cyc]$_3$[Gua]$_3^{MeO}$PCD (4 g) was suspended in a dimethyl formamide solution (100 mL) of $CuCl_2$ (0.28 g, 2.1 mmol) and the mixture was shaken at 20 rpm for 1 day at 25 °C. The resulting resin ([Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD) was washed with dimethyl formamide, water, and acetone, and then dried for 1 day *in vacuo*. IR: 1646 and 1510 (N−H bend), 1200−1000 cm^{-1} (C−N stretch, C−O stretch).

[Cyc]$_2^{Cl}$PCD. This polymer was prepared through attachment of cyclen to PCD by the method described above for the preparation of [Cyc]$_3$[Gua]$_3^{Cl}$PCD. IR: 1512 (N−H bend), 1160−1100 cm^{-1} (C−N stretch).

[Cyc]$_2^{MeO}$PCD. This polymer was prepared from [Cyc]$_2^{Cl}$PCD by the method described above for the preparation of [Gua]$_3^{MeO}$PCD. IR: 1512 (N−H bend), 1160−1100 (C−N stretch), 1089 cm^{-1} (C−O stretch).

[Cu(II)Cyc]$_2^{MeO}$PCD. This polymer was prepared from [Cyc]$_2^{MeO}$PCD by the method described above for the preparation of [Cu(II)Cyc]$_3$-[Gua]$_3^{MeO}$PCD. IR: 1510 (N−H bend), 1200−1000 cm^{-1} (C−N stretch, C−O stretch).

Measurements. Distilled and deionized water was used for preparation of buffer solutions. The swelling of dry beads of the Cu(II)-containing PCD derivatives in water was complete within a few minutes and floating beads sank in the buffer solution upon swelling. In a typical kinetic run, ca. 0.3 mL of buffer solution containing bovine serum γ-globulin (Gbn) (purchased from Sigma; used without further purification) and the swollen beads of a PCD derivative was stirred with a tiny magnetic bar. The stirring speed was controlled with a tachometer. Temperature was controlled within ±0.1 °C with a circulator. The degree of cleavage of Gbn in the presence of the PCD derivatives was measured by SDS-PAGE gel electrophoresis. Densities of the electrophoretic bands were analyzed with software purchased from Jandel Corporation. Buffers (0.05 M) used for the kinetic measurements were glycine (pH 3), sodium acetate (pH 4.5), 4-morpholineethanesulfonic acid (pH 6), 4-(2-hydroxyethyl)-1-pipera-

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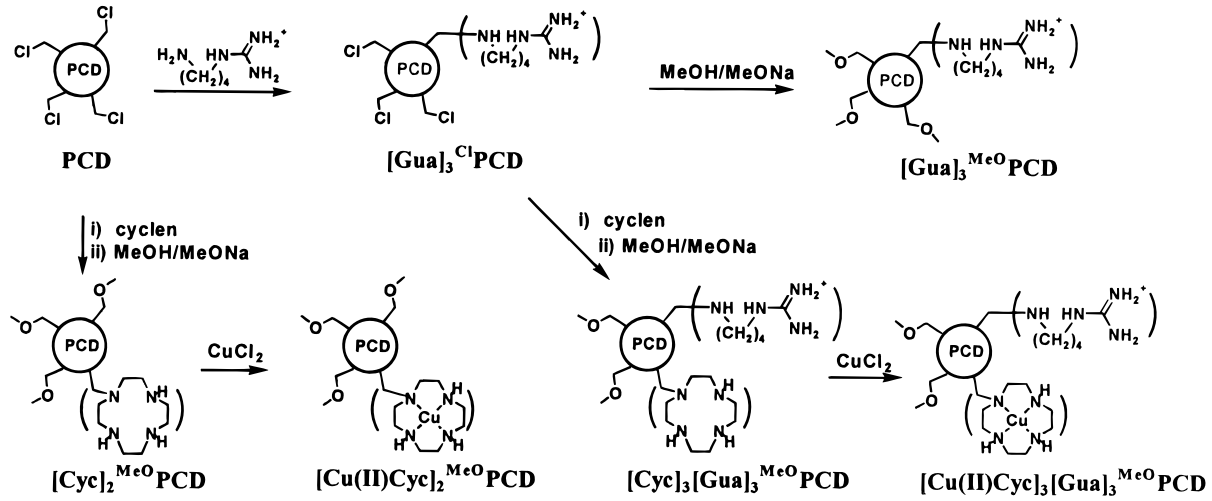
Scheme 1



zineethanesulfonic acid (pH 7, 8), and boric acid (pH 9, 10). pH measurements were carried out with a Dongwoo Medical DP-880 pH/Ion meter. GC analysis was performed with a HP 5890 series II gas chromatograph. UV−vis spectra were taken with a Beckman DU 68 spectrophotometer. HPLC analysis of *N*-labeled peptide mixtures, amino acid analysis, and *N*-terminal sequencing of peptides by Edman degradation were performed by Korea Basic Science Research Institute with a Waters PicoTag System and a Perkin Elmer Procise 491 model. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) measurements were carried out with a Voyager PerSeptive linear model. Inductively coupled plasma absorption emission spectroscopy (ICP-AES) measurements were performed with a Shimadzu ICPS-1000IV model. IR spectra were obtained as KBr pellets with a Bruker FT-IR IFS 48 model. Scanning electron micrographs were taken with a Jeol JSM-840A model.

Results

PCD was prepared by suspension copolymerization of chloromethylstyrene (7:3 mixture of *m* and *p* isomers) and divinylbenzene. A previous study revealed that PCD with a high mechanical strength and a high specific surface area (ca. 3.7 $m^2\ g^{-1}$ when 100 vol % of cyclohexane and 2.5 mol % of divinylbenzene were used) was obtained by using 100−120 vol % of cyclohexane as a diluent.[8]

PCD beads (0.10 g) were suspended in 5 mL of acetonitrile containing triethylamine (140 μL; 1.00 mmol) and chlorobenzene (30 μL). The mixture was stirred at 40 °C for 5 days and the amount of triethylamine consumed was checked at an interval of 24 h by gas chromatography. Chlorobenzene served as the internal standard. When the amount of divinylbenzene used in the copolymerization was 2 mol % relative to that of chloromethylstyrene, the amount of chloromethylphenyl moieties of PCD that reacted with triethylamine was 90−95 mol % of chloromethylstyrene used in the copolymerization. When similar measurements were made with PCD beads prepared with 5 mol %, 7 mol %, or 10 mol % divinylbenzene, the fraction of the reactive benzyl chloride moiety was 80−85, 65−70, or 45−50 mol %, respectively, of the initially used chloromethylstyrene. In the subsequent experiments, PCD beads cross-linked with 2 mol % divinylbenzene were used.

As summarized in Scheme 1, various derivatives of PCD were prepared by substitution of the chlorides of PCD with agmatine and/or cyclen. The unreacted chloromethylphenyl moieties of the resulting resins were treated with methoxide ion. By adding Cu(II) ion, the cyclen moieties attached to PCD were converted to the Cu(II) complex. The PCD derivatives thus prepared include $[Gua]_3^{MeO}$PCD, $[Cyc]_2^{MeO}$PCD, $[Cu(II)Cyc]_2^{MeO}$PCD, $[Cyc]_3[Gua]_3^{MeO}$PCD, and $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD. In the nomenclature of the PCD derivatives, Gua, Cyc, and Cu(II)-Cyc represent catalytic elements such as the guanidinium ion, the cyclen moiety retaining metal binding ability, and the Cu(II) complex of cyclen, respectively, attached to the polymer skeleton. The subscripts stand for the contents of the respective functional groups expressed in terms of residue mol % relative to the chloromethylstyrene units of PCD. Superscript MeO indicates that the remaining chloro groups are treated with methoxide ion.

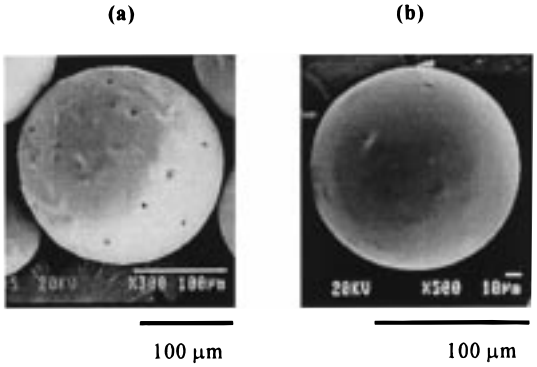


Figure 1. . Scanning electron micrograph of typical beads of PCD (a) and $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD (b).

The scanning electron micrographs of PCD and $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD are illustrated in Figure 1. IR spectra of the PCD derivatives showed characteristic peaks for the functional groups present in the polymers as indicated in the Experimental Section.

The content of the guanidinium ion introduced to PCD was estimated by examining the degree of complexation of the *p*-nitrobenzoate anion. The formation constant (K_f) for the complex formed by adsorption of a guest molecule (G) on a binding site (BS) of an insoluble polymer support can be defined as k_{ad}/k_{de}. Here, k_{ad} and k_{de} are the rate constants for the adsorption and the desorption processes, respectively, as indicated by the scheme of eq 1. Analogous definition is made in the Langmuir isotherm for adsorption of gas molecules on solid surfaces.[12] In addition, complexation of the guest molecule to a binding site can be assumed to be independent of succeeding

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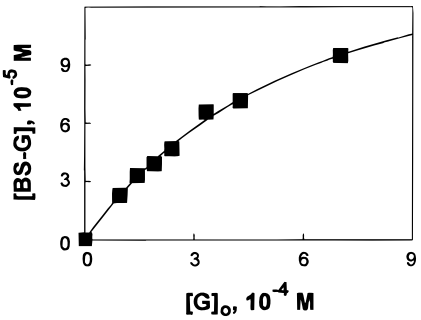

Figure 2. The plot of [BS-G] against $[G]_o$ for the complexation of *p*-nitrobenzoate to $[Gua]_3^{MeO}$PCD at pH 7.50 and 25 °C. The concentration of *p*-nitrobenzoate uncomplexed to the resin was measured spectrophotometrically after the reaction mixture was shaken at the speed of 120 rpm for 1 day. The theoretical curve was constructed on the basis of eq 2.

bindings, again by analogy with the Langmuir isotherm. As the expression of the equilibrium mixture, eq 2 is derived from eq 1. Here, θ is the fractional coverage of the binding site, [BS-G] and $[BS]_o$ are the concentration of BS-G and the total concentration of BS, respectively, obtainable if the insoluble polymer is assumed to be dissolved, and $[G]_o$ is the total concentration of G.

$$\mathrm{BS} + \mathrm{G} \underset{k_{de}}{\overset{k_{ad}}{\rightleftharpoons}} \mathrm{BS\text{-}G} \tag{1}$$

$$\theta = [\mathrm{BS\text{-}G}]/[\mathrm{BS}]_o = [\mathrm{G}]/([\mathrm{G}] + 1/K_f)$$

$$= ([\mathrm{G}]_o - [\mathrm{BS\text{-}G}])/\{([\mathrm{G}]_o - [\mathrm{BS\text{-}G}]) + 1/K_f\} \tag{2}$$

The plot of [BS-G] against $[G]_o$ is illustrated in Figure 2 for the complexation of *p*-nitrobenzoate to $[Gua]_3^{MeO}$PCD. Analysis of the data according to eq 2 with a nonlinear regression program led to $K_f = (2.14 \pm 0.25) \times 10^3\ M^{-1}$ in water (pH 7.50 and 25 °C). The content of guanidinium in $[Gua]_3^{MeO}$PCD was estimated from the value of $[BS]_o$ as 2.56 ± 0.17 mol % relative to the chloromethylstyrene monomer incorporated into PCD. When calculated from the results of elemental analysis, the molar content of the guanidinium residues was estimated as 3.90%. Agreement between the contents based on the two methods appears to be acceptable, considering the lower accuracy of estimation based on the results of elemental analysis and the possibility that access of *p*-nitrobenzoate to some guanidinium moieties is sterically blocked.

The content of the Cu(II) complex of cyclen in $[Cu(II)Cyc]_2^{MeO}$PCD or $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD was determined by measuring the amount of Cu(II) released after treatment of the PCD derivative with 1.5 N HNO_3. ICP-AES analysis of the released Cu(II) revealed that the content of Cu(II)-cyclen is 1.74 mol % for $[Cu(II)Cyc]_2^{MeO}$PCD or 2.55 mol % for $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD relative to the chloromethylstyrene monomer incorporated into PCD. On the basis of the results of elemental analysis of $[Cyc]_2^{MeO}$PCD or $[Cyc]_3[Gua]_3^{MeO}$PCD, the content of cyclen is estimated as 3.52 mol % for $[Cyc]_2^{MeO}$PCD or 3.80 mol % for $[Cyc]_3[Gua]_3^{MeO}$PCD. The discrepancy between the contents estimated by the two methods may be ascribed in part to multiple attachment of some of the cyclen moieties to PCD losing its affinity toward Cu(II) ion due to the distortion in the geometry of the chelating sites.

The amounts of functional groups attached to PCD can be expressed in terms of milliequivalents per gram of resin (mequiv/g). Then, the contents of the Cu(II)-cyclen moiety in $[Cu(II)Cyc]_2^{MeO}$PCD and $[Cu(II)Cyc]_2[Gua]_3^{MeO}$PCD are 0.114 and 0.160 mequiv/g, respectively. The content of the guanidinium moiety that actively recognizes *p*-nitrobenzoate is 0.159 mequiv/g for $[Cu(II)Cyc]_2[Gua]_3^{MeO}$PCD.

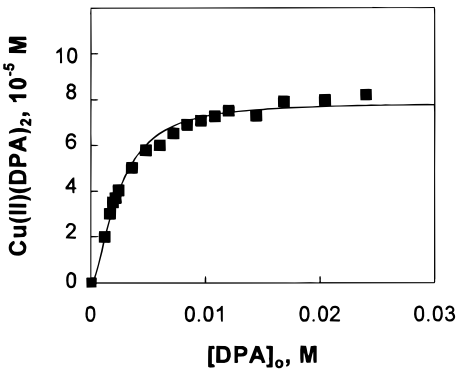

Figure 3. The plot of $[Cu(II)(DPA)_2]$ against $[DPA]_o$ (total concentration of DPA) for abstraction of the Cu(II) ion from $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD with DPA at pH 7.00 and 25 °C. The concentration of Cu(II) ion initially bound to the polymer was 1.02×10^{-4} M. The concentration of $Cu(II)(DPA)_2$ was measured by ICP-AES after the reaction mixture was shaken at 120 rpm for 1 day. The theoretical curve was constructed on the basis of eq 3.

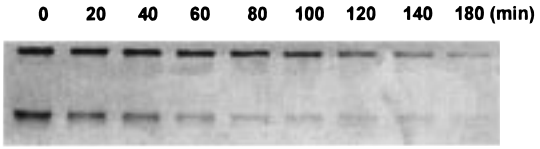

Figure 4. Results of electrophoresis performed with Gbn incubated with $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD at 4 °C and pH 7.00 ($S_o = 8.54 \times 10^{-7}$ M, $C_o = 8.24 \times 10^{-4}$ M). The upper and the lower bands correspond to the heavy and the light chains, respectively, of Gbn.

The formation constant for the Cu(II) complex of the PCD derivative also can be defined by eq 1 where G is Cu(II) and BS is the chelating site for the Cu(II) ion. The value of log K_f for the Cu(II) site of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD was measured by a competition experiment using dipicolinic acid (DPA) as the chelating reagent of Cu(II) ion. As indicated by eq 3, DPA dissolved in the buffer solution can abstract Cu(II) ion from the polymer to form $Cu(II)(DPA)_2$. The data (Figure 3) obtained for the exchange reaction were analyzed with an expression for eq 3 derived by analogy with eq 2. Analysis of the data revealed that 22.7 ± 0.1 % of the Cu(II) ions of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD were bound to the resin so strongly that they were not extracted by DPA. For the remaining Cu(II) ions complexed to the resin, the value of k_1/k_{-1} was estimated. From the value of k_1/k_{-1} thus obtained and the formation constant reported in the literature[13] for $Cu(II)(DPA)_2$, log K_f for the Cu(II) site of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD was estimated as 14.66 ± 0.04 at pH 7.00 and 25 °C.

$$\mathrm{Cu(II)\text{-}BS} + 2\mathrm{DPA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{BS} + \mathrm{Cu(II)(DPA)_2} \tag{3}$$

As illustrated in Figure 4, electrophoresis (SDS-PAGE)[14] revealed facile cleavage of both the heavy (MW 50 kDa) and the light (MW 25 kDa) chains of Gbn during incubation with $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD or $[Cu(II)Cyc]_2^{MeO}$PCD.[15] The rate of protein cleavage was measured by following the decrease in the density of the bands corresponding to the heavy and the

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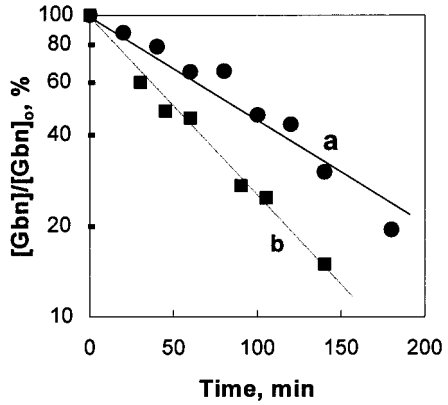

Figure 5. The plot of log [Gbn]/[Gbn]$_o$ against incubation time for the data illustrated in Figure 4. The relative concentrations of the light (a) and the heavy (b) chains were estimated by measuring the densities of the respective bands.

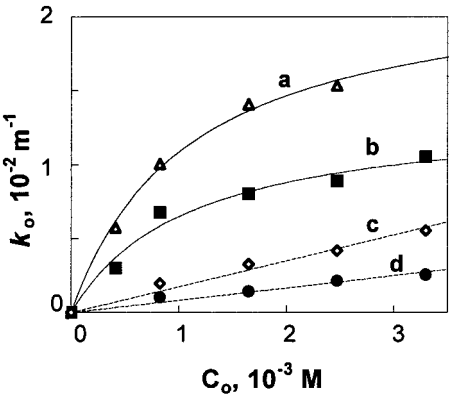

Figure 6. The plot of k_o against C_o for the hydrolysis of the light (a; △) and the heavy (b; ■) chains of Gbn catalyzed by [Cu(II)Cyc]$_3$-[Gua]$_3^{MeO}$PCD and of the light (c; ◇) and the heavy (d; ●) chains of Gbn catalyzed by [Cu(II)Cyc]$_2^{MeO}$PCD at 4 °C and pH 7.00. Curves a and b are constructed on the basis of kinetic parameters summarized in Table 1. Slopes (k_{cat}/K_m) of lines c and d are 1.75 ± 0.08 and 0.83 ± 0.05 m^{-1} M^{-1}, respectively.

light chains. As illustrated in Figure 5, pseudo-first-order kinetic behavior was observed up to at least 2 half-lives, from which the pseudo-first-order rate constant (k_o) was estimated.

Since bulky molecules tend to diffuse slowly to the reactive sites in the resin beads,[6f] stirring speed may affect the kinetics of Gbn cleavage. The effects of stirring speed on the rate of cleavage of the heavy and the light chains of Gbn by the PCD derivatives were examined by using a magnetic stirrer equipped with a tachometer. When examined with sample runs using [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD or [Cu(II)Cyc]$_2^{MeO}$PCD, k_o increased as the stirring speed was raised, reaching a plateau value at 800−1200 rpm. The rest of the kinetic studies were performed at the stirring speed of 900 rpm. At this stirring speed, beads of [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD or [Cu(II)-Cyc]$_2^{MeO}$PCD were not broken appreciably during the period needed for kinetic measurement.

Rate data were collected by varying the amount of the catalyst (C_o). In this study, C_o is expressed as the concentration of the Cu(II) complex of cyclen obtainable when the resin is assumed to be dissolved. As illustrated in Figure 6 for the dependence of k_o on C_o, saturation kinetic behavior was observed at pH 4.5−7 for the cleavage of both the heavy and light chains of Gbn by [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD. On the other hand, k_o was proportional to C_o at pH 3 and 8−10.

Kinetics of the cleavage of the heavy and the light chains of Gbn by the PCD derivatives can be analyzed in terms of the Michaelis−Menten scheme (eq 4). Under the conditions of $C_o >> S_o$, pseudo-first-order kinetic behavior is expected with k_o being derived as eq 5. In homogeneous systems under the conditions of $C_o >> S_o$, the quantity followed during kinetic measurements is the sum of the concentrations of free substrate ([S]) and the catalyst−substrate complex ([CS]). In heterogeneous systems such as the reaction of the PCD derivatives, [S] is followed in the kinetic measurement. In both cases, eq 5 is applicable.

$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} C + P \quad (4)$$

$$k_o = k_{cat}C_o/(K_m + C_o) \quad (5)$$

Saturation kinetic data such as those illustrated in Figure 6 were analyzed according to eq 5 with a nonlinear regression program. The values of kinetic parameters thus obtained are summarized in Table 1. When $C_o << K_m$, eq 5 predicts proportionality between k_o and C_o with the proportionality constant of k_{cat}/K_m. The pH profiles of k_{cat}/K_m for the cleavage of the heavy and the light chains of Gbn by [Cu(II)Cyc]$_3$-[Gua]$_3^{MeO}$PCD are illustrated in Figure 7.

Rates of the cleavage of the two chains of Gbn by [Cu(II)-Cyc]$_2^{MeO}$PCD were considerably slower compared with those by [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD as indicated in Figure 6. Thus, the pH dependence of rate data for [Cu(II)Cyc]$_2^{MeO}$PCD was measured at 25 °C whereas that for [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD was measured at 4 °C. For the [Cu(II)Cyc]$_2^{MeO}$PCD-catalyzed cleavage reactions, saturation kinetic behavior was not observed as exemplified by the dependence of k_o on C_o illustrated in Figure 6. Thus, the proportionality constant k_o/C_o corresponds to k_{cat}/K_m. The pH dependence of k_{cat}/K_m values estimated for the cleavage of the two chains of Gbn by [Cu(II)Cyc]$_2^{MeO}$PCD is illustrated in Figure 8.

The pH dependence of k_{cat}/K_m was analyzed with Scheme 2.[16] Here, **CH$_2$**, **CH**, and **C** stand for the catalyst in various ionization states with **CH** being the catalytically active species. The values of $(k_{cat}/K_m)^{lim}$, pK_1, and pK_2 estimated from the nonlinear regression of the pH profiles are summarized in Table 2.

Right after the cleavage of Gbn (2.68×10^{-6} M) in the presence of [Cu(II)Cyc]$_3$[Gua]$_3^{MeO}$PCD (5.15×10^{-3} M) at pH 7.00 was complete as judged by electrophoresis, the supernatant was collected by filtration. Amino acid analysis of the product solution indicated that about 70% of the amino acids included in Gbn were recovered in the product solution. MALDI-TOF MS measurement was performed with the product solution revealing that the peptides present in the mixtures were not larger than 5 kDa. In a separate experiment, the product solution was treated with phenyl isothiocyanate to label the primary amino group of each peptide product. Phenyl isothiocyanate is the reagent used in Edman degradation to convert the primary amino groups of peptides to phenylthiocarbamoyl derivatives. The HPLC chromatogram of the *N*-labeled peptide products is illustrated in Figure 9. In another experiment, *N*-terminal sequencing of the mixture of peptides obtained as the product of the Gbn cleavage was carried out by Edman degradation.

(15) Gbn (MW 150 kDa), which is also called immunoglobulin G, contains two heavy chains and two light chains and is dissociated into the subunits under the conditions of SDS-PAGE.

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Table 1. Values of Kinetic Parameters Estimated by Analysis of Saturation Kinetic Behavior Observed for Hydrolysis of Gbn by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ at 4 °C

	pH		
	4.50	6.00	7.00
	(1) heavy chain		
k_{cat}, m^{-1}	$(1.13 \pm 0.01) \times 10^{-2}$	$(1.55 \pm 0.01) \times 10^{-2}$	$(1.36 \pm 0.15) \times 10^{-2}$
K_m, M	$(1.48 \pm 0.20) \times 10^{-3}$	$(1.43 \pm 0.01) \times 10^{-3}$	$(1.09 \pm 0.33) \times 10^{-3}$
k_{cat}/K_m, m^{-1} M^{-1}	7.62 ± 1.01	10.8 ± 0.07	12.5 ± 3.98
	(2) light chain		
k_{cat}, m^{-1}	$(5.64 \pm 1.20) \times 10^{-2}$	$(6.92 \pm 0.56) \times 10^{-2}$	$(2.26 \pm 0.15) \times 10^{-2}$
K_m, M	$(3.59 \pm 1.17) \times 10^{-3}$	$(3.52 \pm 0.44) \times 10^{-3}$	$(1.08 \pm 0.17) \times 10^{-3}$
k_{cat}/K_m, m^{-1} M^{-1}	15.7 ± 6.1	19.7 ± 2.9	20.9 ± 3.5

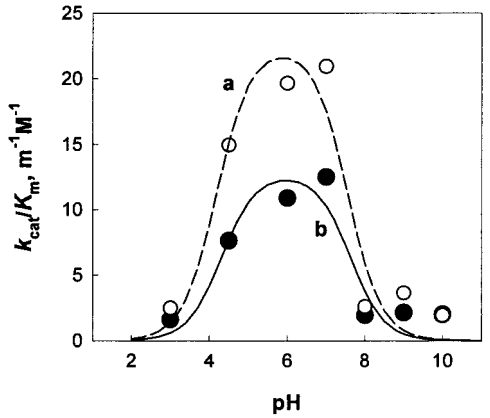

Figure 7. The pH profiles of k_{cat}/K_m for the hydrolysis of the light (a; ○) and the heavy (b; ●) chains of Gbn catalyzed by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ at 4 °C. The theoretical curves were obtained by fitting the data to Scheme 2.

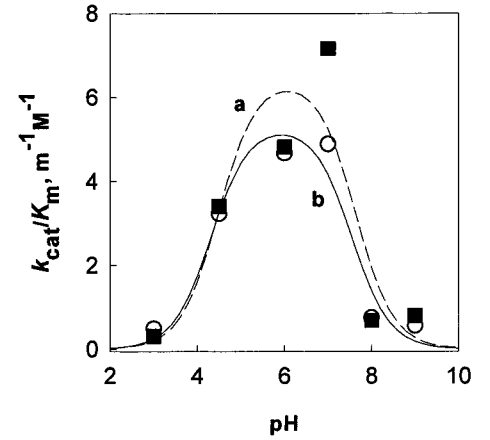

Figure 8. The pH profiles of k_{cat}/K_m for the hydrolysis of the light (a; ■) and the heavy (b; ○) chains of Gbn catalyzed by $[Cu(II)Cyc]_2^{MeO}PCD$ at 25 °C. The theoretical curves were obtained by fitting the data to Scheme 2.

The sequencing data indicated that 4.3% of the amino acids initially contained in Gbn became *N*-terminal amino acid residues of the peptide products. Thus, the average number of amino acids for each peptide product appears to be 23. For the cleavage of Gbn (8.54×10^{-7} M) by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ (4.12×10^{-4} M) at pH 7.00 and 4 °C, exclusion of oxygen and light did not affect the rate appreciably.

When Gbn was incubated with the Cu(II) complex of cyclen at pH 4.5−8 and 25−50 °C, pseudo-first-order kinetic behavior was observed for the cleavage of Gbn with the optimum pH being ca. 7. The kinetic data measured under the conditions of $C_o >> S_o$ are summarized in Table S1. The k_o values were

Scheme 2

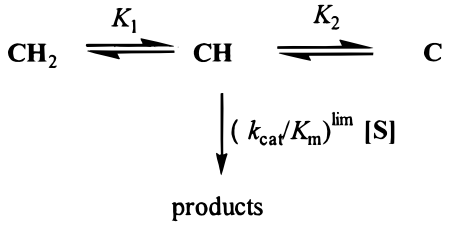

Table 2. Values of Parameters Estimated from Analysis of pH Profiles of k_{cat}/K_m for Hydrolysis of Gbn Catalyzed by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ at 4 °C or $[Cu(II)Cyc]_2^{MeO}PCD$ at 25 °C

	catalyst			
	$[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$		$[Cu(II)Cyc]_2^{MeO}PCD$	
substrate	heavy chain	light chain	heavy chain	light chain
$(k_{cat}/K_m)^{lim}$, m^{-1} M^{-1}	12.8 ± 2.2	22.5 ± 3.3	5.36 ± 0.72	6.47 ± 1.60
pK_1	4.32 ± 0.36	4.19 ± 0.33	4.31 ± 0.27	4.48 ± 0.49
pK_2	7.62 ± 0.35	7.55 ± 0.30	7.55 ± 0.27	7.64 ± 0.51

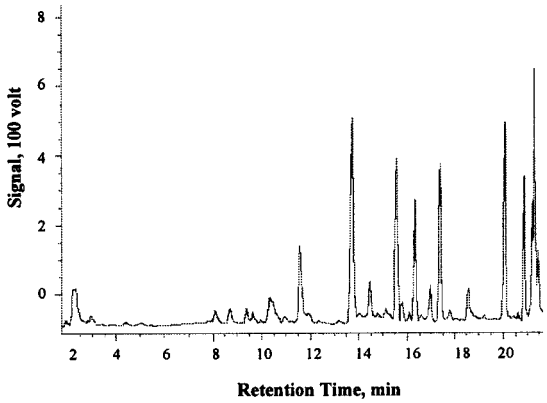

Figure 9. HPLC chromatogram of peptide products separated by filtration after cleavage of Gbn (2.68×10^{-6} M) by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ (5.15×10^{-3} M) at pH 7.00 and 4 °C. The primary amino groups of the peptide products were labeled with phenyl isothiocyanate prior to HPLC analysis. Typical conditions employed by the Waters PicoTag System were used for the elution.

proportional to C_o with the proportionality constant (k_o/C_o) being $(7.20 \pm 0.35) \times 10^{-3}$ m^{-1} M^{-1} for the heavy chain and $(9.33 \pm 0.58) \times 10^{-3}$ m^{-1} M^{-1} for the light chain at pH 7.00 and 25 °C. At 25 °C, rate data for the hydrolysis of Gbn (8.54×10^{-7} M) catalyzed by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ were also measured at pH 7.00. Due to the fast rates, low values (0.1−0.3 mM) of C_o were used. Under these conditions, k_o was proportional to C_o with the proportionality constant ($k_o/C_o = k_{cat}/K_m$) being 62.2 ± 1.2 m^{-1} M^{-1} for the heavy chain and 97.2 ± 1.8 m^{-1} M^{-1} for the light chain.

Discussion

PCD is related to Merrifield's peptide resin[17] since both of them are based on copolymers of styrene and divinylbenzene. In Merrifield's resin, the phenyl groups are partly chloromethylated whereas all of the styrene monomers used in the preparation of PCD are chloromethylated. In PCD, the phenyl groups except for the cross-linking units contain chloromethyl groups, which can be transformed to various functional groups.

To design artificial enzymes, several catalytic elements including catalytic groups and binding sites are to be incorporated. By substituting chloromethylphenyl groups with a variety of functional groups, catalytic centers and binding units can be readily attached to PCD. Moreover, the microenvironment on PCD can be tailored to adjust its polarity as well as its affinity for various solvents. PCD is a good candidate for the backbone of immobile artificial enzymes due to its highly branched structure, availability of many chloromethylphenyl groups on its surface, and easy modification of chloromethylphenyl groups by nucleophilic substitution reactions as well as large surface areas and high mechanical strengths of its beads.

As the first artificial enzyme built on the backbone of PCD, an effective artificial metalloproteinase was prepared in the present study by attaching the Cu(II) complex of cyclen as the catalytic center and guanidinium ion as the binding unit. The PCD derivatives prepared in the present study were characterized by scanning electron microscopy, IR spectroscopy, elemental analysis, titration of the chloromethylphenyl moiety with triethylamine, complexation of *p*-nitrobenzoate ion to the guanidinium moiety, quantification of the cyclen moieties retaining high affinity for the Cu(II) ion, and determination of log K_f for the Cu(II) binding sites.

When the degree of cross-linkage of the PCD backbone was 2 mol % as expressed by the content of divinylbenzene, up to 95% of the chloromethyl groups underwent substitution with triethylamine. As the degree of cross-linkage is raised, the extent of substitution of the chloromethyl group with triethylamine decreased. This indicates that the chloromethyl group was not affected appreciably during the suspension polymerization conducted in water. It is likely that some of the chloromethyl groups of PCD are sterically protected from the attack by triethylamine especially when the degree of cross-linkage is high. Almost quantitative substitution of the chloromethyl group with triethylamine demonstrates that triethylamine has access to most of the chloromethyl groups of PCD when the degree of cross-linkage is 2%.

Metal ions play versatile roles as Lewis acid catalysts in organic reactions.[9] For example, metal complexes of cyclen derivatives have been used as catalytic centers for hydrolysis of phosphate esters including DNA, RNA, and cAMP.[18] Several metal complexes that catalyze hydrolysis of peptide bonds are reported and the mechanisms of the catalytic peptide hydrolysis are well documented.[9] In this regard, the metal centers created by attachment of the Cu(II) complex of cyclen to PCD can be utilized as catalytic groups for peptide hydrolysis.

Guanidinium ion recognizes anions such as carboxylate or phosphate ester anions. For example, the guanidinium ion of Arg-145 of carboxypeptidase A plays an essential catalytic role by recognizing the carboxylate anion of the substrate.[10] Complexation of the phosphate ester anion or the carboxylate ion by the guanidinium ion in water becomes considerably stronger as the microenvironment is changed from bulk water to micelles, bilayer membranes, or the air−water interface.[11] The ability of the guanidinium ion to bind the carboxylate anion or the phosphate ester anions would be improved at the interface between water and the beads of PCD derivatives. The guanidinium ion therefore can be utilized as binding sites for substrates containing carboxylate or phosphate ester anions such as proteins or nucleic acids.

The surface of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD contains both the hydrophobic character imposed by phenyl groups and the polar nature arising from guanidinium ions and the Cu(II) complexes. This mimics the unique microenvironments of enzyme active sites.[19] Since rates of many organic reactions are sensitive to the properties of the media, the microenvironments of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD may be utilized to promote the effectiveness of guanidinium ions and the metal centers in substrate binding and catalytic conversion.

The product solution separated after the reaction of Gbn with $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD contained a variety of peptides which were smaller than 5 kDa. Quantification of the *N*-terminal residues by Edman degradation and labeling of the *N*-termini of the product peptides prior to taking the chromatogram of Figure 9 indicate that the cleavage of Gbn generated primary amino groups. Exclusion of oxygen and light did not affect the cleavage reaction. These results indicate that the cleavage of Gbn in the presence of PCD derivatives is hydrolysis of the peptide bonds.[20]

The k_o values[21] for hydrolysis of Gbn catalyzed by the derivatives of the Cu(II)-cyclen complex were estimated from the plot of log [Gbn]/$[Gbn]_o$ against time (Figure 5), which did not deviate considerably from the pseudo-first-order behavior up to at least 2 half-lives. This can be taken to indicate the absence of considerable product inhibition under the experimental conditions even though Gbn is cleaved into many small pieces. The pH profiles (Figures 7 and 8) of k_{cat}/K_m for the heavy and the light chains of Gbn have similar shapes for both of the PCD derivatives. When the pH profiles were analyzed according to the method used in enzymology,[16] the p*K* values summarized in Table 2 were obtained. The acidic and the basic limbs of pH profiles are characterized by p*K* of about 4.3 and 7.6, respectively. The pH profiles of k_{cat}/K_m for enzymatic reactions provide information on the catalytic functional groups of the enzyme uncomplexed with the substrate as well as those of uncomplexed substrate. Similarly, the p*K* values estimated from the pH profiles illustrated in Figure 7 or 8 are related to functional groups of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD or $[Cu(II)Cyc]_2^{MeO}$PCD, respectively, as well as Gbn.

The activity of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD for the hydrolytic cleavage of the heavy and the light chains of Gbn is greater than that of $[Cu(II)Cyc]_2^{MeO}$PCD by 12−15 times as judged by k_{cat}/K_m measured at 4 °C and pH 7.00. Saturation kinetic behavior is manifested by $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD at pH 4.5−7, indicating much stronger complexation of Gbn to $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD compared with $[Cu(II)Cyc]_2^{MeO}$PCD. The greater proteolytic activity of $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD is in part related to the more favorable complexation of Gbn (smaller K_m). The more favorable complexation is due to the presence of guanidinium ions on $[Cu(II)Cyc]_3[Gua]_3^{MeO}$PCD which would recognize carboxylate anions of the substrate and act as effective binding sites.

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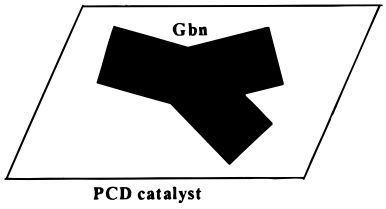
Comparison of the second-order rate constant (k_o/C_o) measured at 25 °C and pH 7.00 reveals that the Cu(II)-cyclen moiety of $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ is more reactive than the Cu(II) complex of cyclen by 9000−10000 times toward the two chains of Gbn. In addition, the Cu(II)-cyclen moiety of $[Cu(II)Cyc]_3^{MeO}PCD$ is more reactive than the Cu(II) complex of cyclen by 700−800 times toward the two chains of Gbn at 25 °C and pH 7.00 (Figure 8 and Table S1). For $[Cu(II)Cyc]_3^{MeO}PCD$ or $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$, only the Cu(II)-cyclen moieties located in sufficiently wide areas on the surface of the resin would be exposed to approach by Gbn. Then, the degree of activation of the Cu(II) complex of cyclen upon attachment to the resins should be considerably greater than that (10^4-fold) estimated above. The microenvironment of Cu(II)-cyclen moieties in the gel phase of the polymer surface contains both hydrophobic and ionic character. The remarkable activation of the Cu(II)-cyclen moiety upon attachment to the PCD derivatives may be in part related to the unique medium properties, although detailed analysis of the medium effects is not possible at present.[21]

The half-life for peptide bonds under the conditions of spontaneous hydrolysis at pH 7 and 25 °C recently has been estimated as about 1000 years.[22] The half-lives of the heavy and the light chains of Gbn are about 60 and 40 min, respectively, at 4 °C in the presence of $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ ($C_o > 3$ mM) at pH 7.00 (Figure 6). The half-life of 40 min at 4 °C corresponds to 10^8-times faster reaction than the half-life of 1000 years at 25 °C. Electrophoresis of the reaction mixtures indicated that only the parent proteins of the heavy and the light chains were detected during the cleavage reaction catalyzed by the PCD derivatives. Thus, any other proteins with sizes large enough for detection by the electrophoretic method did not accumulate appreciably during the reaction. Under the conditions of electrophoresis, only proteins larger than several kDa are detected. Analysis of the reaction products by MALDI-TOF MS revealed that proteins smaller than 5 kDa were obtained from the hydrolysis of the heavy (50 kDa) and the light (25 kDa) chains. Initial cleavage of the heavy and the light chains of Gbn should produce intermediate proteins greater than 25 and 12 kDa, respectively. Thus, the hydrolysis of intermediate proteins into peptides smaller than 5 kDa is much faster than the hydrolysis of the parent proteins.

Gbn (150 kDa) would occupy a large region when bound on the surface of the PCD derivatives as illustrated by **I**. This



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region may be considered as the active site. On the basis of the structure of Gbn,[23] the area of an active site is estimated as ca. 7000 Å^2. The area occupied by each monomer of the PCD resin is estimated as ca. 40 Å^2 on the basis of a molecular mechanics calculation (MM+, HyperChem). Since the actual area occupied by the monomer depends on its orientation on the surface of the PCD resin, the average value for the effective area occupied by each monomer may be approximated as 20 Å^2. Then, about 350 monomers totaling more than 50 kDa are included in an active site. Even such a large active site contains only ca. 9 guanidinium ions and ca. 9 Cu(II)-cyclen residues on the average.

The chance that several guanidinium ions of the active site interact with different carboxylate groups of Gbn simultaneously is not high. It is also unlikely that several Cu(II)-cyclen moieties of the active site interact with different amide groups of Gbn simultaneously, especially when Gbn retains its original quaternary structure. Several peptide bonds of Gbn bound to the active site may be exposed to attack by each Cu(II)-cyclen unit. In this regard, a 10^8-fold difference between the half-life observed for Gbn cleavage by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ and that for spontaneous hydrolysis of peptides may somewhat overestimate the degree of catalysis in peptide hydrolysis by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$. The number of peptide bonds of a Gbn molecule interacting with the catalytic centers of an active site of the resin is, however, not large and the breakdown of the intermediate proteins is faster than the cleavage of parent molecule of Gbn. It is reasonable, therefore, to estimate that the degree of acceleration in the hydrolysis of a peptide bond of Gbn achieved by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ is not considerably smaller than 10^8-fold.

Several homogeneous synthetic artificial enzymes[20,24] and catalytic antibodies[25] with proteinase activity have been reported. The monoclonal catalytic antibody prepared with a phosphinate hapten exhibited optimum activity at pH 9.5. The k_{cat} measured with an amide substrate at pH 9 and 37 °C was 1.65×10^{-7} s^{-1}.[25a] Thus, the half-life is 49 days when the substrate is fully complexed to the active site of the catalytic antibody. A much more improved antibody catalyst for amide hydrolysis has been elicited very recently by a joint hybridoma and combinatorial antibody library approach.[25b] The k_{cat} measured with a primary amide substrate at pH 9 and 25 °C was 5×10^{-5} s^{-1} for this new antibody. This corresponds to a half-life of 4 h when the substrate is fully complexed to the active site. The half-lives for the amide hydrolysis catalyzed by the antibodies are much longer than that (10−30 min at pH 4.5−7 and 4 °C when the substrate is fully complexed to the active site; Table 1) of the light chain of Gbn hydrolyzed by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$. The fastest protein cleavage recorded so far with artificial proteinases is the cleavage of chymotrypsin by a coordinatively polymerized bilayer membrane prepared in this laboratory, which achieved a half-life as short as 3 min at 4 °C and pH 5.5−9.5.[20b] This is several times faster than the hydrolysis of Gbn by $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$. In terms of utility in practical applications, however, $[Cu(II)Cyc]_3[Gua]_3^{MeO}PCD$ is much more useful than the artificial enzyme based on the bilayer membrane due to the immobile nature of the former as well as the intrinsic instability of bilayer membranes.

Complex formation with Gbn by $[Cu(II)Cyc]_3(Gua)_3^{MeO}PCD$ and fast hydrolysis of the complexed Gbn demonstrate the high potential of PCD as the backbone of immobile artificial

(21)) Kinetic data for the hydrolysis of Gbn in the presence of the PCD-based catalysts were collected under the conditions of $C_o >> S_o$ which allowed estimation of values of kinetic parameters such as k_{cat}, K_m, and k_{cat}/K_m. In practical applications employing columns packed with immobilized catalysts, the condition of $C_o >> S_o$ would be met. Then, kinetic information derived under the condition of $C_o >> S_o$ is applicable to such devices.

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enzymes.[26] Active sites of the PCD-based artificial enzymes can be designed better if techniques are developed for positioning two or more catalytic elements in close proximity on the backbone. By choosing the constituents of the active site of the immobile artificial enzyme properly, various types of chemical transformation including synthetic reactions can be catalyzed. Further elaboration of the geometry of active sites can achieve reproduction of major characteristics of enzymatic action such as regioselectivity or enantioselectivity as well as complex formation with substrates and a high degree of rate acceleration.

(26) The PCD derivatives obtained by addition of Zn(II), Ni(II), or Fe(III) ion to $[Cyc]_3[Gua]_3^{MeO}$PCD did not manifest catalytic activity for cleavage of Gbn. For organic reactions catalyzed by metal ions acting as Lewis acids, remarkable changes in catalytic efficiency have been frequently observed when the metal ions or the ligands of the metal centers are varied. Often, the changes in catalytic efficiency are unaccountable and unpredictable.[9a]

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Supporting Information Available: Table S1 summarizing bimolecular rate constants for the hydrolysis of Gbn by the Cu(II) complex of cyclen at 25−50 °C and pH 4.5−8 (1 page; print/PDF). See any current masthead page for ordering information and Web access instructions.

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