Account

Immobile Artificial Metalloproteases

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Effective artificial metalloproteases have been designed by using cross-linked polystyrene as the backbone. Artificial active sites comprising Cu(II) complexes as the catalytic site and other metal centers or organic functionalities as binding sites were synthesized. The activity of Cu(II) centers for peptide hydrolysis was greatly enhanced on attachment to polystyrene. By placing binding sites in proximity to the catalytic centers, the ability to hydrolyze a variety of protein substrates at selected cleavage sites was improved. Thus far, the most advanced immobile artificial proteases have been obtained by attaching the aldehyde group in proximity to the Cu(II) complex of cyclen.

Key Words : Artificial enzymes, Artificial proteases, Metal catalysis, Peptide hydrolysis, Immobilized catalysts

Introduction

Enzymes are the most effective catalysts that exist in Nature, and design of catalysts mimicking the catalytic principles of enzymes is among great challenges of modern chemistry.[1,2] Catalytic antibodies are examples of semi-synthetic artificial enzymes.[3-6] Fully synthetic molecules have been also designed as enzyme mimics by using either peptidic[7,8] or nonpeptidic[9-15] molecules.

In the early stage of designing artificial enzymes, the main objective was to reproduce major characteristics of enzymatic action such as complex formation with substrates, large degrees of rate acceleration, and high selectivity. To design more elaborated artificial enzymes, attempts were later made to overcome limitations of enzymes such as instability to heat, incompatibility with organic solvents, inapplicability to abiotic reactions, and too narrow selectivity.

Following the principles of enzymatic catalysis, an effective enzyme-like catalyst should be able to form a complex with the substrate readily, and the complex should undergo rapid chemical transformation. In the resulting complex, several catalytic components should take productive positions to maximize cooperativity among them and the effective concentrations of the catalytic groups towards the reaction site should be sufficiently high. Moreover, the conformational freedom of the molecular framework should be controlled to maintain the productive structure. Chemical transformations in enzyme-substrate complexes often take place in hydrophobic microenvironments. It is necessary, therefore, to control the microenvironment of the complex formed between an artificial enzyme and its substrate. Construction of such a catalyst-substrate complex with a small molecular framework would not be easy.[16]

Nature has adopted polypeptides as the backbones of enzymes and constructed active sites on those backbones. By using the macromolecular backbones, Nature is able to tune the positions and the reactivity of catalytic elements in enzyme-substrate complexes. A better mimicry of enzymatic action would be, therefore, obtained by adopting macromolecular systems for catalyst-substrate complexes.

Synthetic polymers have been used as backbones of artificial enzymes catalyzing reactions of macromolecular or small substrates.[9-15,17-38] For macromolecular catalytic systems mimicking enzymatic action, it is not easy to fully characterize the structure of the catalyst-substrate complexes and to correctly determine the mechanisms of the catalytic processes. As with catalytic antibodies, therefore, strategies to design the artificial active sites and catalytic outcome of the resulting artificial enzymes are important at the current stage in the area of synthetic artificial enzymes employing macromolecular catalytic systems.

We have been involved in synthesis of enzyme-like catalysts for hydrolysis of peptide bonds of proteins. We chose hydrolysis as the target reaction since only one molecule is involved as the substrate except the water molecule. We selected peptide bonds as the targets since protein hydrolysis is important in the era of genomics and proteomics and peptide bonds are quite stable with the half-

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life[39,40] of spontaneous hydrolysis at pH 7 and 25 °C being 500-1000 years.

Metalloproteases use metal ions as the key catalytic groups. Metal ions can coordinate to the carbonyl oxygen of an amide group, polarizing the carbonyl group and facilitating attack by hydroxide ion.[41-44] Metal-bound hydroxide ions are potent nucleophiles for peptide cleavage.[41-44] Metal-bound water molecules can act as general acids in the rate-determining expulsion of amines from the tetrahedral intermediates.[43-45] Often, a single metal center can perform several catalytic roles.[42-44] When organic functional groups are exploited as catalytic groups for peptide hydrolysis, it is necessary to achieve collaboration among two or more catalytic groups. On the other hand, the active site of an artificial protease can be designed by using a single metal center as the catalytic group.

Early studies in the area of metal-promoted peptide cleavage employed metal complexes in combination with oxidoreductive additives to achieve either hydrolytic[46] or oxidative[47-49] cleavage of the target protein. The Fe(III) complex of EDTA quickly cleaves a peptide bond when tethered to a target protein leading to the formation of hydrolysis products.[46] The Fe(III) complex, however, uses H_2O_2 and ascorbic acid as coreactants. Target-selective oxidative cleavage of proteins by metal complexes such as Cu(II)EDTA or Fe(III)EDTA has been reported previously.[47,48] These reactions, however, are not useful for therapeutic or industrial purposes since they require additives such as mercaptoethanol or dithiothreitol. In addition, the reagents based on Cu(II)EDTA or Fe(III)EDTA should be added at least in the stoichiometric amounts.

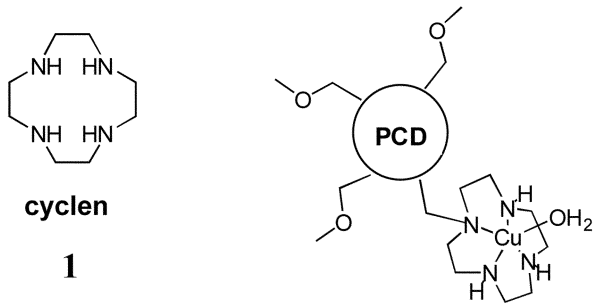
Several kinds of metal complexes have been examined for their ability to catalyze hydrolysis of peptide bonds without using additives,[50-57] but most of them had low catalytic rates unless they were tethered to peptide substrates especially under physiological conditions. Proteolytic action by Pd(II) complexes untethered to proteins has been reported, but they are mostly active at acidic pHs.[52,54,56]

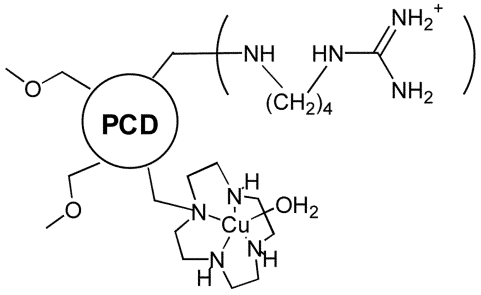
Our studies on artificial proteases have been carried out towards two goals: synthetic proteases hydrolyzing essentially all proteins with high cleavage-site selectivity and synthetic proteases cleaving only the target protein in the presence of many other proteins. This Account describes our efforts made towards immobile artificial metalloproteases with broad substrate selectivity and high cleavage-site selectivity.

Enhancement of Proteolytic Activity of Cu(II) Center on Attachment to Polystyrene. To design an effective artificial metalloprotease operating at near physiological pHs, it is necessary to raise the catalytic activity of metal centers substantially. We attempted to enhance the intrinsic reactivity of the metal centers by changing the microenvironment.

In enzyme-substrate complexes, polar interactions such as hydrogen-bonding and dipole-dipole interactions as well as electrostatic interactions between the substrate and the enzyme contribute significantly to the stabilization of the transition states.[58] Those polar interactions are enhanced in the hydrophobic microenvironments provided by the enzyme. If the microenvironment of the catalytic center of an artificial metalloprotease is modified, the proteolytic activity of metal complexes may be enhanced. The easiest way to obtain hydrophobic microenvironment in water is to attach the catalytic center to a synthetic polymer.

When the Cu(II) complex of cyclen (Cu(II)Cyc) was attached to cross-linked polystyrene, the proteolytic activity of Cu(II)Cyc was enhanced remarkably.[23] We prepared a derivative of polystyrene (poly(chloromethylstyrene-co-divinylbenzene): PCD) in which all of the styryl residues contained chloromethyl groups. By the substitution of chloro groups of PCD with various nucleophiles, PCD derivatives **1** and **2** were prepared. γ-Globulin was effectively hydrolyzed upon incubation with **1** and **2**. The rates for hydrolysis of γ-globulin by Cu(II)Cyc itself dissolved in water were also measured. Comparison of rate data collected at the same catalyst concentrations revealed that the proteolytic activity of Cu(II)Cyc was enhanced by up to 10^4-times upon attachment to the polystyrene. Considering that only a small fraction of Cu(II)Cyc moieties is present on the open surface on PCD and participate in hydrolysis of γ-globulin, the degree of activation should be substantially greater than 10^4-fold.





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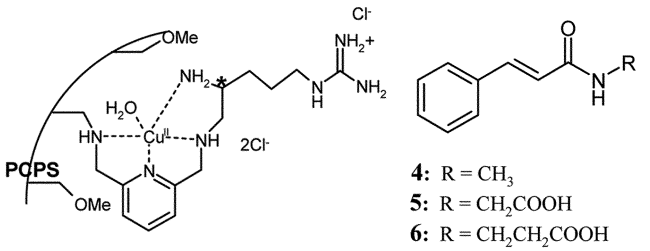
Site-Selective Amide Hydrolysis by Immobile Artificial Peptidase with Active Site Comprising Cu(II) Center and Guanidinium Group. Since reactive metal centers were secured, it was subsequently attempted to achieve substrate selectivity in amide hydrolysis by the metal complexes. The artificial active site of **3** was constructed on the surface of partially chloromethylated cross-linked polystyrene (PCPS) by stepwise modification of the polymer.[30] Here, the active site was chiral since L-Arg was used to introduce the guanidinium portion. Several cinnamoyl amide

derivatives (**4**-**7**) were tested as substrates for **3**. Neutral amide **4** was not hydrolyzed upon incubation with **9**, but carboxyl-containing amides **5**-**7** were effectively hydrolyzed by **3** with the optimum activity observed at pH 9, as shown by the kinetic data illustrated in Figure 1. Both acetyl L-Phe and acetyl D-Phe were also hydrolyzed by **3** and a small (1.5) enantio-selectivity was observed as the catalyst was chiral.



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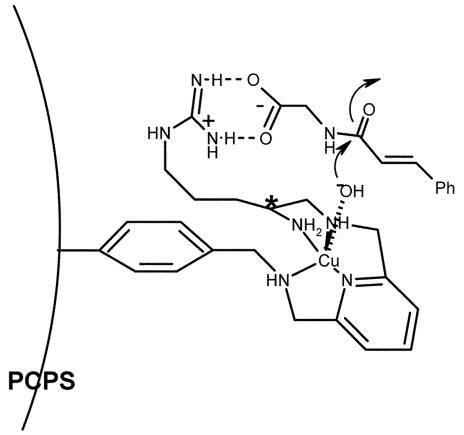
4: R = CH_3
5: R = CH_2COOH
6: R = CH_2CH_2COOH
7: R = $CH_2CH_2CH_2COOH$

In the proposed mechanism (**8**), the carboxylate anion of **5**-**7** or acetyl Phe is recognized by the guanidinium ion and the Cu(II) center subsequently hydrolyzes the amide group. Both the electrostatic interaction between carboxylate and guanidinium ions and the Cu(II)-catalyzed amide cleavage would be facilitated by the microenvironment provided by polystyrene.



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In carboxypeptidase A,[59,60] the active-site Zn(II) ion plays essential catalytic roles whereas guanidinium of Arg-145 recognizes carboxylate anion of the substrates, thus making the enzyme an exopeptidase. Important features of carboxypeptidase A reproduced by **3** include essential catalytic action of a metal ion, participation of a guanidinium group in substrate recognition, hydrolysis of unactivated amides, and substrate selectivity toward amide bonds adjacent to carboxylate groups.

Site-Selective Amide Hydrolysis by Immobile Artificial Peptidase with Active Site Comprising Three Convergent Cu(II) Centers. Many enzymes contain two or more metal ions in the active site, exploiting collaboration among the metal centers in the catalytic action. Examples of multi-

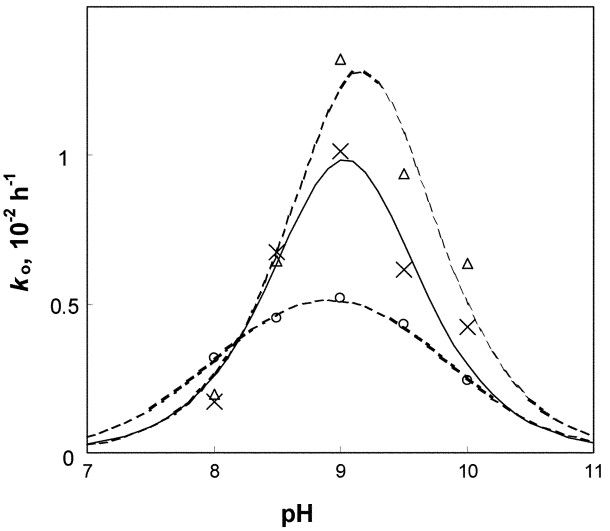
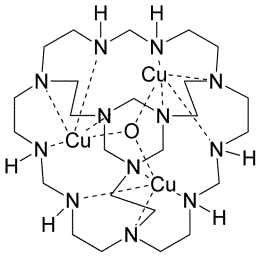


Figure 1. pH dependence of k_o for hydrolysis of **5** (Δ), **6** (X), and **7** (O) (S_o = 1.96 × 10^{-4} M) promoted by **3** (C_o = 1.08 mM) at 50 °C. For the polystyrene-based artificial enzymes, C_o is expressed as the concentration of the active site obtainable when the resin is assumed to be dissolved.

nuclear metallo-enzymes catalyzing hydrolysis of acyl derivatives and related compounds are methionine aminopeptidase,[61] metallo-β-lactamase,[62] proline dipeptidase (prolidase),[63] urease,[64] and agmatinase.[65] In addition, there are a large number of multinuclear metalloenzymes that catalyze several other types of reactions such as nucleic acid hydrolysis, synthetic transformations, or oxidation-reduction.

An effective multinuclear artificial metalloenzyme would be obtained if an artificial active site comprising two or more proximal metal centers is designed. A trinuclear artificial metallopeptidase was prepared[28] by using **9**. Upon treatment of **9** with excess NaH, at least three of the six N-H protons



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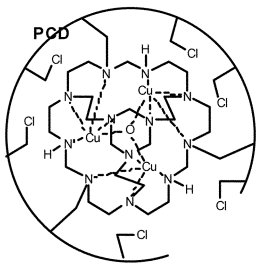

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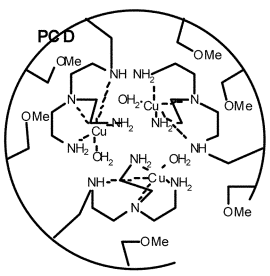

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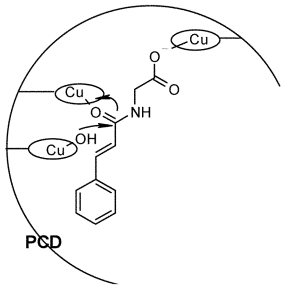

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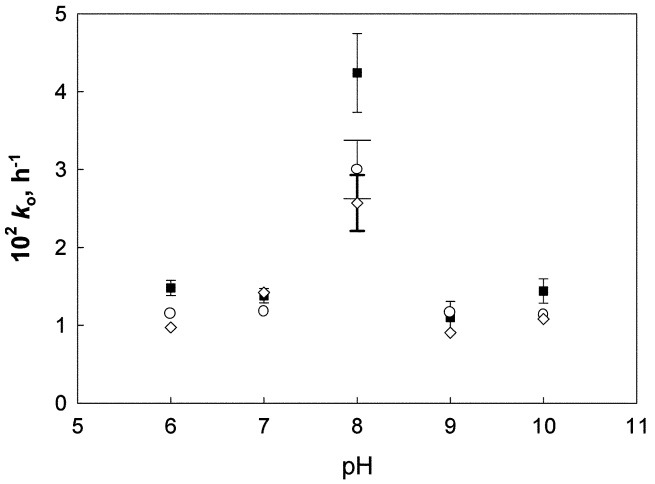


Figure 2. pH dependence of k_o for the hydrolysis of **5-7** (S_o = 1.96 × 10^{-4} M: ■, **5**; ○, **6**; ◇, **7**) catalyzed by **11** (C_o = 5.80 × 10^{-4} M) at 50 °C.

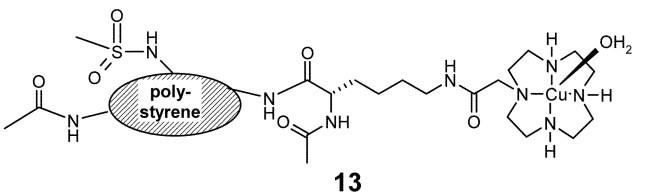
were deprotonated. By mixing the anion of **9** with PCD, **9** was attached to PCD. In **10**, **9** is connected to PCD by triple attachment. After the chloro groups of **9** were reacted with methoxide anion, the resulting resin was treated with acid and then with $CuCl_2$ to produce **11**. The artificial active site in **11** contains three convergent Cu(II) complexes of tris(2-aminoethyl)amine. On incubation with **11**, neutral amide **4** was not affected whereas carboxyl-containing amides **5-7** were effectively hydrolyzed, as shown by the kinetic data summarized in Figure 2. Mechanism of **12** was proposed to account for the substrate selectivity manifested for the carboxyl-containing amides.

The kinetic data were analyzed in terms of Michaelis-Menten scheme (eq 1). Parameter k_{cat}/K_m stands for the reactivity of the catalyst (C) toward the substrate (S) and k_{cat} represents that of the complex formed between the catalyst and the substrate (CS). The k_{cat} value estimated for the amide hydrolysis by **3** or **11** was >> 0.1 h^{-1} at pH 8.5 and 50 °C or >> 0.2 h^{-1} at pH 8 and 50 °C, respectively. This may be compared with the k_{cat} of 0.18 h^{-1} (at the optimum pH of 9 and 25 °C) measured with a catalytic antibody[66] with peptidase activity elicited by a joint hybridoma and combinatorial antibody library approach in the hydrolysis of an amide substrate.

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS \xrightarrow{k_2} C + P \qquad (1)$$

Site-Selective Hydrolytic Cleavage of Protein by Immobile Artificial Peptidase Containing Active Site Built with Catalytic Module Containing Two Cu(II)-Binding Ligands. Catalyst **11** manifested both catalytic activity and substrate selectivity in the hydrolysis of small peptides. The metal centers of the artificial active site of **11** were utilized both in substrate recognition and in catalytic conversion: one metal center recognized the carboxylate group of the substrate and other metal centers cleaved the peptide bond. Little information is available, however, for the structure of the active site obtained by using the bowl-shaped molecule. In addition, it is not possible to synthesize a variety of artificial multinuclear metalloenzymes by the method of transferring catalytic elements confined in a prebuilt cage to a synthetic polymer.

To develop a methodology applicable to designing a wide range of multinuclear polymeric artificial metalloenzymes, we prepared active sites by attaching a molecular entity comprising various catalytic elements with precisely defined structure ("a catalytic module") to a polymeric backbone. Thus, new multinuclear polymeric artificial metalloproteases (**13-15**) were synthesized by preparation of catalytic modules containing one, two, or four metal-chelating sites followed by attachment of the modules to a polystyrene and addition of metal ions to the chelating sites.[37]



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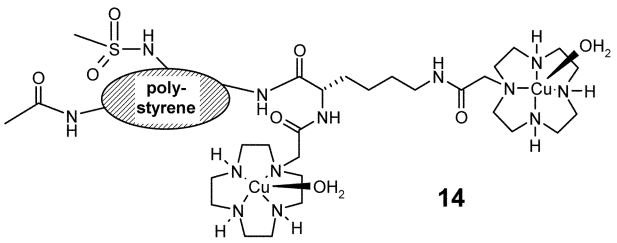

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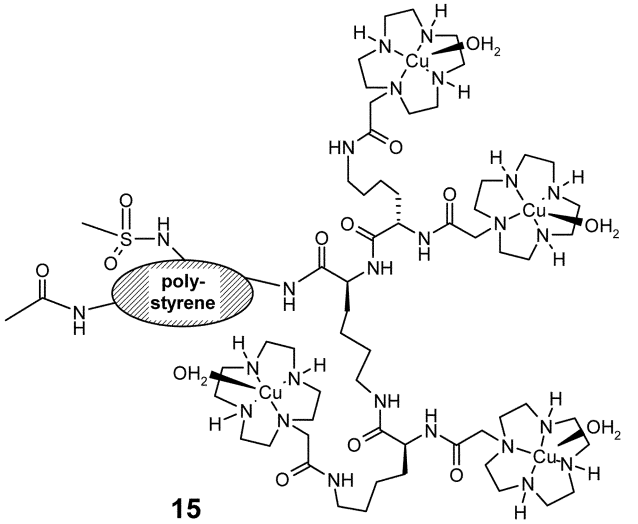

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Proteolytic activity of **13-15** was examined by using horse heart myoglobin, bovine serum γ-globulin, or bovine serum albumin as the substrate. γ-Globulin and albumin were not cleaved appreciably over the period of 24 h at pH 7-10 and 37 °C or 50 °C. On the other hand, considerable proteolytic

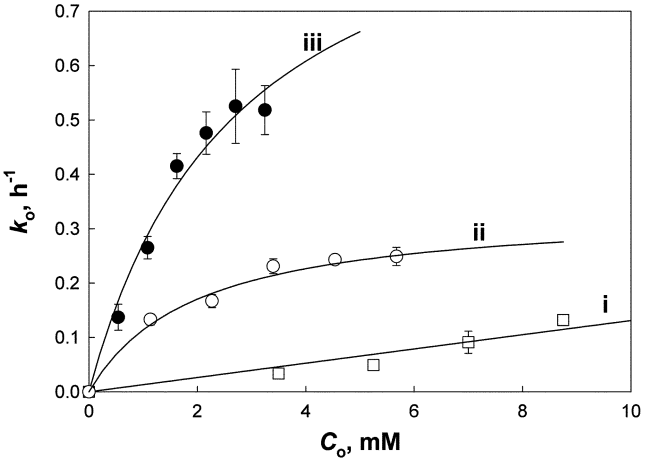


Figure 3. The plot of k_o against C_o for the hydrolysis of myoglobin catalyzed by **13** at pH 9.0 and 50 °C (i), **14** at pH 9.0 and 50 °C (ii), or **15** at pH 9.0 and 37 °C (iii).

activity was observed with myoglobin.

For reactions obeying the Michaelis-Menten Scheme (eq 1), pseudo-first-order kinetic behavior is expected under the conditions of $C_o >> [CS]$, with k_o being derived as eq 2. Kinetic data for the cleavage of myoglobin by **13**-**15** were collected by varying C_o. Saturation kinetic behavior was observed at pH 8-10 for the cleavage of myoglobin by **14** or **15**. For the kinetic data collected with **13**, k_o was proportional to C_o indicating $K_m >> C_o$ (proportionality constant $= k_{cat}/K_m$) (eq 2). Figure 3 illustrates kinetic data obtained for **13**-**15** at their optimum pHs. The kinetic data for **15** were measured at 37 °C whereas those for **13** and **14** were obtained at 50 °C. Considering the temperature difference, the ratio of k_{cat}/K_m values can be estimated as 1 : 13 : 100 for **13**-**15** and the ratio of k_{cat} as 1 : 10 for **14** and **15**. As the catalytic group density of the module is increased in the polystyrene-based catalysts, up to 100-fold enhancement is achieved in the reactivity of the catalyst. An enzymatic active site has a high value of catalytic group density with the catalytic groups placed in highly productive positions. In the polystyrene-based catalysts, catalytic activity was considerably improved simply by raising the catalytic group density without deliberate positioning of the catalytic groups.

$$k_o = k_{cat}C_o / (K_m + C_o)$$
$$\text{where } k_{cat} = k_2 \text{ and } K_m = (k_{-1} + k_2)/k_1 \quad (2)$$

As illustrated in Figure 4, intermediate proteins accumulated in amounts detectable by the electrophoresis (SDS-PAGE) during the cleavage of myoglobin by **14**. On the other hand, accumulation of intermediate proteins was not detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF MS) or SDS-PAGE when myoglobin was incubated with **13** or **15**. MALDI-TOF MS taken for the intermediate protein mixture obtained by incubation of myoglobin with **14** is illustrated in Figure 5. Figure 5 indicates the presence of protein fragments obtained by cleavage of myoglobin at two different positions: peaks with

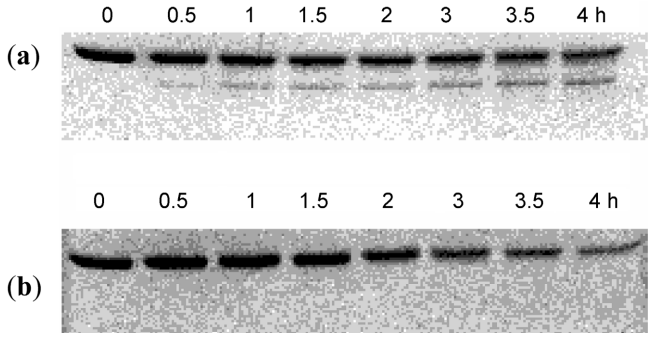


Figure 4. Results of SDS-PAGE performed on myoglobin incubated with the PS-based catalysts: (a) **14** ($C_o = 2.3 \times 10^{-3}$ M) at pH 9.0 and 50 °C, (b) **15** ($C_o = 1.1 \times 10^{-3}$ M) at pH 9.0 and 37 °C.

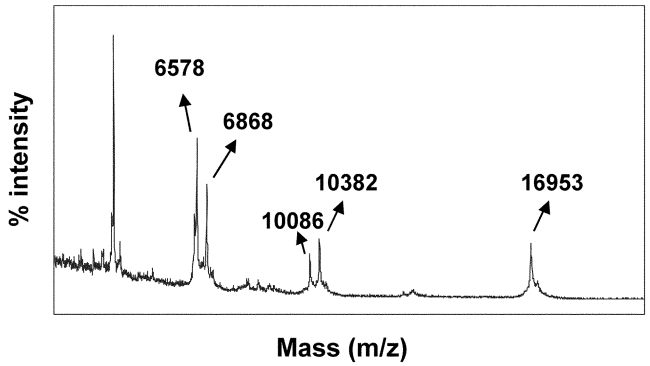


Figure 5. MALDI-TOF MS spectrum of degradation products obtained by incubation of myoglobin (1.2×10^{-5} M) with **14** ($C_o = 2.3 \times 10^{-3}$ M) at pH 9.0 and 50 °C for 4 hours.

m/z of 6868 and 10086 originate from cleavage of myoglobin (m/z = 16953; the heme group dissociates during measurement of MALDI-TOF MS) at a single position and those of 6577 and 10382 from cleavage of myoglobin at another position.

In order to characterize the intermediate protein fragments formed during cleavage of myoglobin with **14**, the electrophoretic band newly appearing below the band of myoglobin (Figure 4a) was excised from SDS-PAGE gels and extracted. *N*-Terminal sequencing of the extracted protein mixture was carried out by Edman degradation. In order to identify the *C*-terminal residues of the intermediate proteins, the reaction mixture separated from the catalyst was treated with carboxypeptidase A followed by MALDI-TOF MS measurement. The sizes of new protein fragments obtained after treatment of a cleavage product with carboxypeptidase A provide information for amino acid sequence at the *C*-terminus of the cleavage product. Based on the results of the *N*-terminal and the *C*-terminal sequencing experiments as well as the molecular weights of the protein fragments indicated by MALDI-TOF MS, the two initial cleavage sites of myoglobin for the action of **14** were identified as Gln(91)-Ser(92) and Ala(94)-Thr(95). Results of the *N*-terminal sequencing and the *C*-terminal sequencing of the intermediate proteins indicate that the amino and carboxyl groups are generated after cleavage of myoglobin with **14**

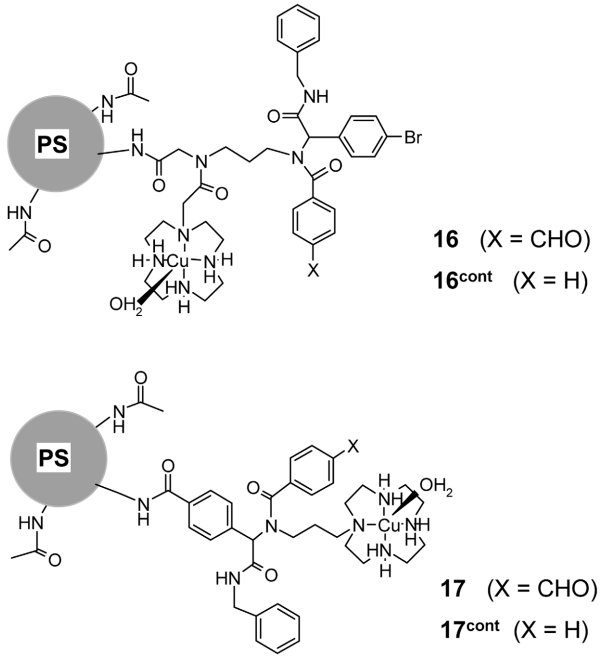
and demonstrate the hydrolytic nature of the protein cleavage by **14**.

High site selectivity manifested by **14** may be attributed to anchorage of one Cu(II)Cyc of the catalyst to a site on myoglobin and interaction of the other Cu(II)Cyc center with the scissile peptide bond. Various functional groups of myoglobin may be considered as the anchorage site for the cleavage at Gln(91)-Ser(92) or Ala(94)-Thr(95). Myoglobin contains more than 50 functional groups of amino acid residues such as Asp, Glu, Lys, His, Met, and Tyr that can coordinate to the Cu(II) center of the polystyrene-based catalysts. The heme carboxylate group might be considered as the most probable anchorage site. The myoglobin molecule might have some structural features that guide foreign molecules to the heme pocket. The heme carboxylate, which is located at the gate of entrance into the heme pocket, may serve as the initial contact point for the catalysis. With a Cu(II)Cyc moiety of **14** anchored to the heme carboxylate, interaction of the other Cu(II)Cyc with the peptide group of Gln(91)-Ser(92) or Ala(94)-Thr(95) agrees with the size of the linker connecting the two Cyc moieties. Interaction of the catalyst with myoglobin at Gln(91)-Ser(92) or Ala(94)-Thr(95) does not involve steric interaction with other parts of the protein when examined with molecular modeling programs (HyperChem and Sculpt).

Site-Selective Hydrolytic Cleavage of a Broad Range of Proteins by Immobile Artificial Peptidase Containing Active Site Built with Catalytic Module Containing Cu(II)-Binding Ligand and Aldehyde Group. Although high site selectivity for the initial cleavage of a protein substrate was achieved with **14**, **14** failed to cleave other common proteins such as γ-globulin or albumin. One of the next challenges in the area of synthetic artificial proteases is to design catalysts that hydrolyze a wide range of protein substrates (broad substrate selectivity) by cleaving the substrate at selected positions on the polypeptide backbone (high site selectivity). In order to possess both the broad substrate selectivity and the high site selectivity as digestive proteases[67] or proteasomes,[68] the artificial proteases should be able to form complexes with a variety of protein substrates effectively and to cleave peptide bonds at selected positions in the resulting complexes.

For this purpose, the aldehyde group was employed as the binding site of the artificial proteases because the aldehyde group can form imine bonds with the ε-amino groups of Lys residues exposed on the surface of proteins. Since the imine bonds are readily hydrolyzed, the artificial protease equipped with an active site containing the aldehyde group may be able to form complexes with a variety of proteins reversibly. If a catalytic group with high proteolytic activity is positioned in proximity to the aldehyde group, the catalytic group may cleave a peptide bond in vicinity to the Lys residue of the substrate complexed to the artificial protease. The cyclen-aldehyde conjugates built on the backbone of polystyrene are indicated by **16** and **17**.[38] As the control catalysts lacking the aldehyde groups, **16**^{cont} and **17**^{cont} were also prepared.

Proteolytic activity of **16**, **16**^{cont}, **17**, and **17**^{cont} was



examined by using horse heart myoglobin, bovine serum γ-globulin, bovine serum albumin, human serum albumin, chicken egg white lysozyme, and chicken egg ovalbumin as the substrates. Examples of the kinetic data are illustrated in Figure 6. The values of k_{cat}, K_m, and k_{cat}/K_m for the cleavage of the protein substrates by **16**, **16**^{cont}, **17**, and **17**^{cont} are summarized in Table 1. As demonstrated by the kinetic data summarized in Table 1, **16** and **17** effectively degraded all of the six protein substrates. The values of k_{cat}/K_m for myoglobin degradation by **16** and **17** are 20-80 times greater than that by **14**. Catalysts **16** and **17** are even more effective for γ-globulin and bovine serum albumin, since these proteins were not cleaved by **14**. On the other hand, **16**^{cont} and **17**^{cont} lacking the aldehyde groups in the active sites exhibited negligible proteolytic activity. By positioning the aldehyde group in proximity to Cu(II)Cyc attached to a

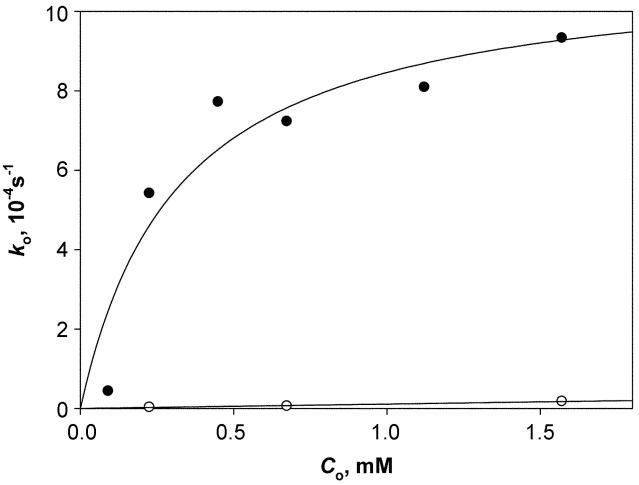


Figure 6. The plot of k_o against C_o for the hydrolysis of myoglobin catalyzed by **16** (●) and **16**^{cont} (○) at pH 9.5 and 50 °C.

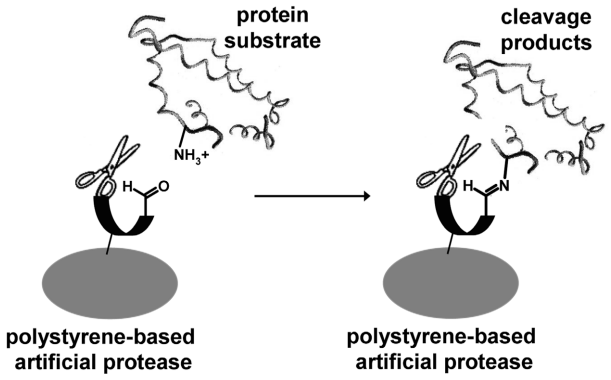
Table 1. Values of kinetic parameters for the cleavage of various protein substrates by **16** and **17** at 50 °C and at the optimum pH

substrate	catalyst	pH	k_{cat} (10^{-4} s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)[a]
myoglobin	**16**	9.5	11	0.32	3.5 (0.005)
myoglobin	**17**	9.5	6.7	0.77	8.7 (0.003)
γ-globulin	**16**	9.5	22	1.3	1.7 (0.008)
γ-globulin	**17**	9.5	7.8	0.57	1.4 (0.005)
bovine serum albumin	**16**	9.5	8.0	0.92	0.87 (0.005)
bovine serum albumin	**17**	9.5	8.7	1.2	0.73 (0.005)
human serum albumin	**16**	9.5	18	0.79	2.3 (0.008)
human serum albumin	**17**	9.0	6.7	1.1	0.61 (0.006)
lysozyme	**16**	9.5	6.2	0.13	4.8 (0.002)
lysozyme	**17**	9.5	6.0	1.3	0.46 (0.002)
ovalbumin	**16**	9.5	25	1.2	2.1 (0.003)
ovalbumin	**17**	9.5	15	2.2	0.68 (0.002)

[a]Values in parentheses are k_{cat}/K_m measured with the respective control polymer (**16**^{cont} or **17**^{cont}) under the same conditions. Accuracy of theses values is low.

crosslinked polystyrene, both broad substrate selectivity and high proteolytic rate are achieved.

The broad substrate selectivity and high proteolytic rate can be attributed to the imine formation between the aldehyde group of the artificial protease and the amino groups of the protein substrate as schematically indicated by **18**. Essentially all globular proteins possess Lys residues which provide ammonium cations on the protein surfaces. Imine formation between the Lys-amino group of the protein substrate and the aldehyde group of the polystyrene-based artificial protease would anchor the protein on polystyrene. The broad substrate selectivity manifested by **16** and **17** is attributable to the anchorage of the protein substrates through imine bonds. Upon formation of the covalent complex, cleavage of the polypeptide backbone of the bound protein substrate could occur more readily since the reaction between the catalytic Cu(II) center and the scissile peptide bond becomes entropically more favorable.



18

Attempts were made to trap the imine intermediates with $NaB(OAc)_3H$ which is known to reduce imines much faster than aldehydes. Since reductive amination with $NaB(OAc)_3H$ is usually carried out under weakly acidic conditions[69] and **16** and **17** are deactivated under acidic conditions, the trapping experiments were carried out at pH 6.0. When myoglobin (S_o = 4.9 μM) was degraded by **16** (C_o = 0.45 mM) at pH 6.0 and 50 °C, k_o was 6.8×10^{-6} s^{-1}. After disappearance of myoglobin was complete, 88% of the total amino acid residues initially introduced by myoglobin were recovered in the buffer solution. When 5 mM $NaB(OAc)_3H$ was initially introduced to the reaction mixture, k_o was 1.3×10^{-4} s^{-1} and only 15% of the total amino acid residues were recovered in the buffer solution. When the same reaction was carried out first without $NaB(OAc)_3H$ to complete the degradation of myoglobin, $NaB(OAc)_3H$ was added to the degradation mixture, and the mixture was incubated for 3 days, 72% of the total amino acid residues were recovered in the buffer solution. The enhanced rate for disappearance of the protein substrate is consistent with trapping of the imine intermediate with the borohydride reagent. Reduced amount of amino acid residues recovered from the reaction mixture incubated with the borohydride reagent also supports trapping of imine intermediate.

During incubation of a protein substrate with **16** or **17**, aliquots of the buffer solution were taken to measure MALDI-TOF MS. An example is illustrated in Figure 7. Disappearance of myoglobin, formation and decay of some intermediate proteins, and accumulation of product proteins are shown in the MALDI-TOF MS. The content of each reaction mixture isolated at various time intervals was partially separated by HPLC to obtain fractions containing fewer numbers of cleavage products. Each cleavage product

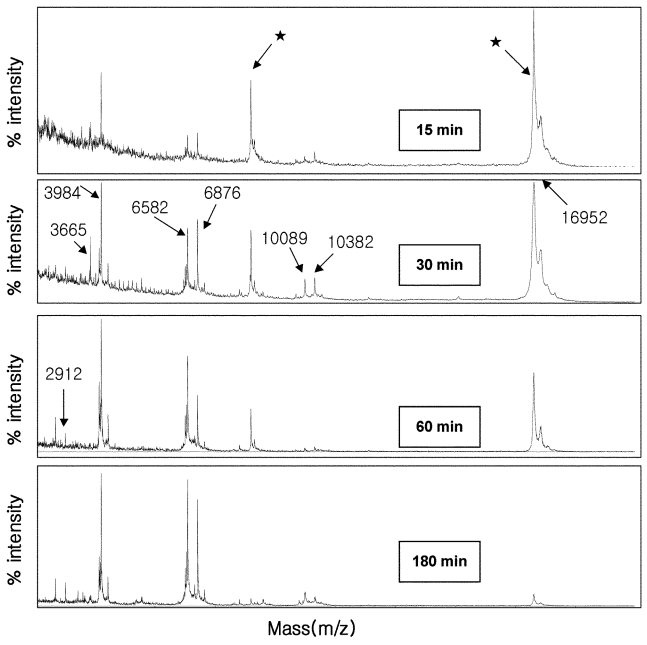


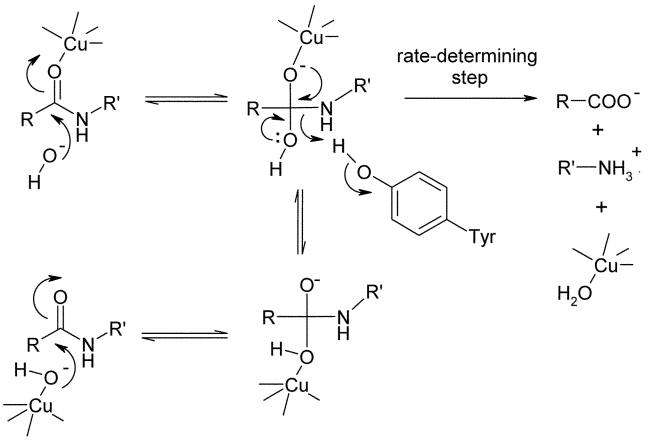
Figure 7. MALDI-TOF MS obtained during incubation of myoglobin (S_o = 4.9 μM) with **16** (C_o = 0.45 mM) at pH 9.5 and 50 °C. The peaks labeled with asterisks (*m/z* = 16952 and 8476) are those of myoglobin. The peaks with *m/z* of 10382, 10089, 6876, 6582, 3984, 3663, and 2912 are identified as Gly(1)-Ala(94), Gly(1)-Gln (91), Ser(92)-Gly(153), Thr(95)-Gly(153), Ser(117)-Gly(153), Gly (1)-Phe(33), and Ser(92)-His(116), respectively.

contained in the fraction was subjected to structural analysis by treatment with carboxypeptidase A or trypsin followed by MALDI-TOF MS measurement. As mentioned above, The results obtained with carboxypeptidase A and MALDI-TOF MS provide information for amino acid sequence at the *C*-terminus of the cleavage product. When the *C*-terminal amino acid of a cleavage product is Gly, Asp, Glu, Pro, or Arg, the cleavage product resists the action of carboxypeptidase A. Then, treatment of the cleavage product with trypsin can provide a different kind of information on the structure of the cleavage product.

The structures of the seven cleavage products and the positions of four cleavage sites were identified for the action of **16** on myoglobin. Among the seven cleavage products, five were also identified in the action of **17**. For both **16** and **17**, Gln(91)-Ser(92) and Ala(94)-Thr(95) were the major initial cleavage sites. Myoglobin contains 19 Lys residues. Although molecular mechanics calculations were carried out by using molecular modeling programs (HyperChem and Sculpt), it was not possible to identify the Lys residue of myoglobin that was anchored on the aldehyde group of the catalytic module prior to the initial peptide cleavage. This was mainly due to the flexibility of the artificial active site comprising Cu(II)Cyc and the aldehyde group. If the flexibility of the protein, especially the long side chains of the Lys residues, is taken into consideration, it is more difficult to identify the anchorage site. The catalytic center, however, selectively attacks peptide bonds of Gln(91)-Ser(92) or Ala(94)-Thr(95) in spite of the flexibility of the artificial active site comprising Cu(II)Cyc and the aldehyde group. Interestingly, Gln(91)-Ser(92) and Ala(94)-Thr(95) were also the initial cleavage sites in the degradation of myoglobin by **14**.

The selective cleavage of Gln(91)-Ser(92) and Ala(94)-Thr(95) in the action of **14**, **16**, and **17** despite the structural differences in the catalytic modules implicates that some structural features may assist the peptide cleavage at Gln (91)-Ser(92) and Ala(94)-Thr(95). Examination of the crystallographic structure of myoglobin suggested that the phenol group of Tyr(146) may promote the cleavage of both Gln(91)-Ser(92) and Ala(94)-Thr(95) by acting as an extra catalytic group. The distance between the phenol oxygen of Tyr(146) and the amide nitrogen atom of Ser(92) or Thr(95) is 6.7 Å or 6.2 Å, respectively, in the crystallographic structure.[70] Little steric strain is imposed, however, when the distance between the phenol oxygen atom and either of the two amide nitrogen atoms is reduced to 3 Å.

As summarized in Scheme 1, the Cu(II) center may act as a Lewis acid catalyst to polarize the carbonyl group of the scissile peptide bond and facilitate attack by hydroxide ion at the carbonyl carbon. Alternatively, the Cu(II)-bound hydroxide ion may act as the nucleophile. In either case, the rate-determining step would be the expulsion of amine moiety from the tetrahedral intermediate, which requires protonation of the amine nitrogen by the specific acid or a general acid.[43] The phenol of Tyr(146) may act as the general acid catalyst to accelerate the rate-determining amine expulsion. At pH



Scheme 1. Possible action of Tyr-146 as a general acid catalyst.

9.0-9.5, the optimum pH for the polystyrene-based artificial proteases, the phenol of Tyr is the strongest acid among the organic functionalities present in the protein molecule.

The initial cleavage of myoglobin at Gln(91)-Ser(92) or Ala(94)-Thr(95) involves anchorage of the substrate onto **14**, **16**, or **17** as well as cleavage of peptide bonds by the Cu(II)Cyc moiety of the artificial protease with general acid assistance from the Tyr phenol group provided by the substrate. The optimum pH of 9.0-9.5 reflects the overall efficiency of complexation of the protein by the catalyst, the formation of imine intermediates, and the catalytic action of Cu(II)Cyc combined with hydroxide ion and the Tyr phenol group.

Conclusions

In vivo, digestive proteases and proteasomes mostly hydrolyze unfolded or precleaved proteins. For example, pepsin cleaves proteins unfolded in the acidic medium of stomach producing protein fragments, which are cleaved further by other proteases in the intestine. Proteasomes, the main enzymes of the non-lysosomal pathway of protein degradation in cells of higher organisms, cleave cellular proteins unfolded with ATP.[68] In the industrial application of artificial proteases,[71,72] however, it is desirable to hydrolyze a variety of proteins without addition of denaturing agents. Hydrophilic residues such as ammonium or carboxylate anions are exposed on the surface of undenatured globular proteins. To synthesize artificial proteases recognizing undenatured protein molecules, we chose to design recognition sites targeting the ammonium groups exposed on the surface of protein substrates. As we expected, artificial proteases **16** and **17** cleaved all of the protein substrates tested in the present study by using both the aldehyde and the Cu(II)Cyc moiety in the active site. Furthermore, the artificial proteases manifested high cleavage site selectivity.

The immobile artificial proteases can overcome thermal, chemical, and mechanical instabilities of natural proteases. Broad substrate selectivity, high proteolytic rate, and high cleavage-site selectivity are the three major objectives in

designing artificial proteases applicable to protein industry. By introducing an auxiliary binding group in proximity to the Cu(II)Cyc attached to polystyrene, remarkable improvement has been achieved in those three major goals. By incorporating more catalytic elements to the artificial active site with a better-defined structure, the artificial proteases would become more effective and achieve higher reaction rates and high amino acid specificity.

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