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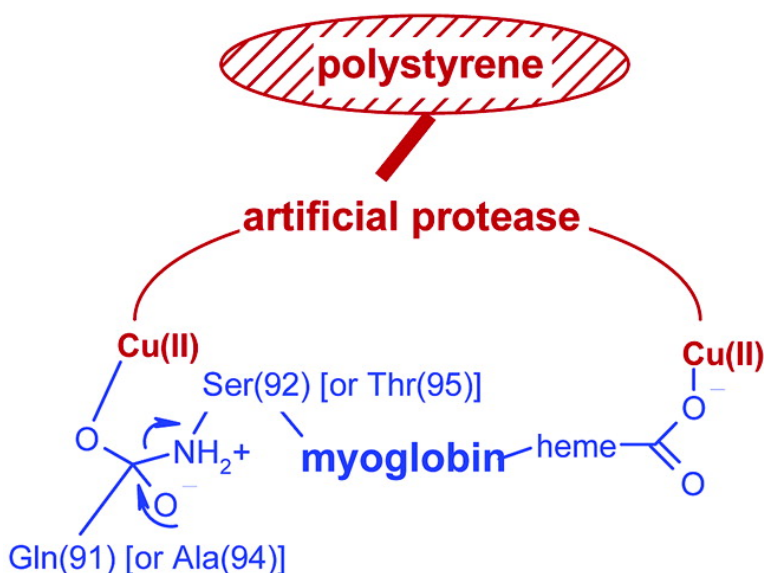
Degradation of Myoglobin by Polymeric Artificial Metalloproteases Containing Catalytic Modules with Various Catalytic Group Densities: Site Selectivity in Peptide Bond Cleavage

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Degradation of Myoglobin by Polymeric Artificial Metalloproteases Containing Catalytic Modules with Various Catalytic Group Densities: Site Selectivity in Peptide Bond Cleavage

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Abstract: Mononuclear, dinuclear, and tetranuclear artificial metalloproteases were prepared by attaching respective catalytic modules containing the Cu(II) complex of cyclen (Cu(II)Cyc) to a derivative of cross-linked polystyrene. The polymeric artificial metalloproteases effectively cleaved peptide bonds of myoglobin (Mb) by hydrolysis. The proteolytic activity increased considerably as the catalytic group density was raised: the ratio of k_{cat}/K_m was 1:13:100 for the mono-, di-, and tetranuclear catalysts. In the degradation of Mb by the dinuclear catalyst, two pairs of intermediate proteins accumulated. One of the two initial cleavage sites leading to the formation of the protein fragments is identified as Gln(91)-Ser(92) and the other is suggested as Ala(94)-Thr(95). On the basis of a molecular modeling study by using the X-ray crystallographic structure of Mb, the site-selectivity is attributed to anchorage of one Cu(II)Cyc unit of the catalytic module to a heme carboxylate of Mb. The high site selectivity for the initial cleavage of a protein substrate and mechanistic analysis of the catalytic action are unprecedented for polymeric artificial enzymes.

Introduction

Synthetic polymers have been utilized as the backbones of artificial enzymes.[1-7] Enzyme-like catalysts built on synthetic polymers would possess chemical, thermal, and mechanical stabilities. In addition, macromolecules can carry enough molecular information for substrate recognition and chemical transformation that are needed for effective catalytic action.[8] Several strategies have been reported for designing active sites of polymeric artificial enzymes.[5-7]

Various organic functional groups present in the side chains of amino acids play key catalytic roles in enzymatic action acting as nucleophiles, general acids, or general bases.[9] The organic functional groups are also exploited in binding of the substrate through various polar or nonpolar interactions with the substrate. Metal ions, metal-bound water molecules, or metal-bound hydroxide ions can play the roles listed above.[10-13] Metal ions

are often more effective than organic functional groups in catalyzing organic reactions. For example, several catalytic systems have been reported for peptide hydrolysis in which the metal centers are either tethered or untethered to the peptide substrates.[10-28] In peptide hydrolysis, metal ions can polarize and activate the carbonyl group of the scissile peptide bond,[10-13] metal-bound hydroxide ion is an effective nucleophile that attacks at the carbonyl carbon,[10-13] and metal-bound water can act as a general acid in expulsion of the leaving amine from the tetrahedral intermediate.[12,20] Often, a single metal center performs several catalytic roles simultaneously.[12,13] Active sites

(1) Klotz, I. M. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; Chapter 2.
(2) Wulff, G. *Angew. Chem., Intl. Ed. Engl.* **1995**, *34*, 1812−1832.
(3) Suh, J. In *Polymeric Materials Encyclopedia; Salamone*, J. C., Ed.; CRC Press: Boca Raton, 1996; 4210−4218 and 8230−8237.
(4) Hodge, P. *Chem. Soc. Rev.* **1997**, *26*, 417−424.
(5) Suh, J. *Adv. Supramolecular Chem.* **2000**, *6*, 245−286.
(6) Suh, J. *Synlett.* **2001**, *9*, 1343−1363.
(7) Suh, J. *Acc. Chem. Res.* **2003**, *36*, 562−570.
(8) Dugas, H. *Bioorganic Chemistry*, 3rd ed.; Springer-Verlag: New York, 1996; p3, 172, and 255.
(9) Silverman, R. B. In *The Organic Chemistry of Drug Design and Drug Action*; Academic Press: San Diego, 1992; pp 103−113.
(10) Sutton, P. A.; Buckingham, D. A. *Acc. Chem. Res.* **1987**, *20*, 357−364.
(11) Chin, J. *Acc. Chem. Res.* **1991**, *24*, 145−152.
(12) Suh, J. *Acc. Chem. Res.* **1992**, *25*, 273−279.
(13) Suh, J. In *Perspectives on Bioinorganic Chemistry*; Hay, R. W., Dilworth, J. R., Nolan, K. B., Eds.; JAI Press: London, 1996; Vol. 3, 115−149.

(14) Chin, J.; Jubian, V.; Mrejen, K. *J. Chem. Soc., Chem. Commun.* **1990**, 1326−1328.
(15) Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10 578− 10 582.
(16) Zhu, L.; Qin, L.; Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218−5224.
(17) Hegg, E. L.; Burstyn, J. N *J. Am. Chem. Soc.* **1995**, *117*, 7015−7016.
(18) Kaminskaia, N. V.; Johnson, T. W.; Kostic, N. M. *J. Am. Chem. Soc.* **1999**, *121*, 8663−8664.
(19) Saha, M. K.; Bernal, I. *J. Chem. Soc., Chem. Commun.* **2003**, 612−613.
(20) Suh, J.; Park, T. H.; Hwang, B. K. *J. Am. Chem. Soc.* **1992**, *114*, 5141− 5146.
(21) Suh, J.; Cho, Y.; Lee, K. J. *J. Am. Chem. Soc.* **1991**, *113*, 4198−4202.
(22) Jang, B.-B.; Lee, K. P.; Min, D. H.; Suh, J. *J. Am. Chem. Soc.* **1998**, *120*, 12 008−12 016.
(23) Suh, J.; Hong, S. H. *J. Am. Chem. Soc.* **1998**, *120*, 12 545−12 552.
(24) Moon, S.-J.; Jeon, J. W.; Kim, H.; Suh, M. P.; Suh, J. *J. Am. Chem. Soc.* **2000**, *122*, 7742−7749.
(25) Suh, J.; Moon, S.-J. *Inorg. Chem.* **2001**, *40*, 4890−4895.
(26) Jeung, C. S.; Kim, C. H.; Min, K.; Suh, S. W.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2401−2404.
(27) Jeung, C.-S.; Song, J. B.; Kim, Y.-H.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3061−3064.
(28) Jeon, J.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Song, J. B.; Suh, J. *Org. Lett.* **2002**, *4*, 4155−4158.

of polymeric artificial enzymes, therefore, have been constructed by using metal complexes[21−28] as well as organic[29−34] functional groups.

Many enzymes contain two or more metal ions in the active site, exploiting collaboration among the metal centers in the catalytic action. Examples of multinuclear metalloenzymes catalyzing hydrolysis of acyl derivatives and related compounds are methionine aminopeptidase,[35] metallo-β-lactamase,[36] proline dipeptidase (prolidase),[37] urease,[38] and agmatinase.[39] 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. Various small molecules with multiple metal centers have been synthesized and tested for catalytic activity in several organic reactions including phosphoester hydrolysis[40] and asymmetric organic reactions such as epoxidation,[41] aldol condensation,[42] and carbonyl reduction.[43] On the other hand, construction of an artificial active site containing two or more metal centers on a polymeric backbone has been seldom attempted.

In an effort to design an artificial active site comprising multiple metal centers on the backbone of synthetic polymers, we have reported transfer of metal-chelating sites confined in a bowl-shaped molecule to a cross-linked polystyrene.[24] The resulting artificial active site contained three moieties of Cu-(II) complex of tris(2-aminoethyl)amine and manifested both catalytic activity and substrate selectivity in the hydrolysis of small peptides. The metal centers of the artificial active site 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 attempted in the present study to prepare active sites by attaching a molecular entity comprising various catalytic elements with precisely defined structure ("a catalytic module") to a polymeric backbone. Thus, it was attempted to synthesize new multinuclear polymeric artificial metalloproteases 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. Previous studies indicated that polymer backbones such as polystyrene provide microenvironments that can enhance the intrinsic reactivity of metal centers in the hydrolysis of peptide or phosphodiester bonds.[22,26,27] In addition, polar interactions such as electrostatic interaction, hydrogen bonding, and dipole−dipole interaction become stronger in more hydrophobic environments, possibly leading to stabilization of substrate-catalyst complexes and the transition states.

With an artificial enzyme obtained by attaching a prebuilt catalytic module to a synthetic polymer, it is easier to interpret catalytic outcome on molecular basis since the structure of the catalytic module is known. Although several effective polymeric artificial enzymes have been designed for protein or DNA hydrolysis with various novel strategies to build artificial active sites,[22,26,27,30−34] detailed mechanistic analysis has not been performed due to lack of information on exact structure of the active sites.

The catalytic element used in construction of the catalytic module in the present study is the Cu(II) complex of cyclen (Cyc). Cu(II)Cyc was chosen in view of the tight binding[44] of Cu(II) ion to Cyc at near neutral pH's and proteolytic[22] activity of Cu(II)Cyc attached to polystyrene derivatives. As the number of the Cu(II)Cyc units present in the catalytic module is increased, the catalytic group density of the active site is raised. Improvement of catalytic activity of the artificial active site through an increase in the catalytic group density would become a new methodology for designing artificial active sites. When the multinuclear metal center participates in cleavage of a protein substrate, some of the metal centers may act as the binding sites to recognize the protein substrate and other metal centers as catalytic groups to hydrolyze peptide bonds. If a certain functional group of the protein substrate is recognized, then some peptide bonds of the substrate may be selectively cleaved. Selectivity in cleavage site is one of the important goals to achieve at the current stage in the area of designing enzyme-mimetic catalysts. In this article, synthesis of the multinuclear artificial metalloproteases, kinetic data for the hydrolytic cleavage of myoglobin (Mb), and site-selectivity of the catalytic action in Mb cleavage are described.


Cyc
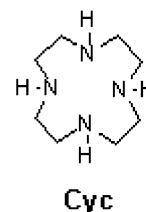
Results

Synthesis of Catalysts. The catalytic modules containing one, two, or four Cyc units were synthesized by attaching Cyc

(29) Suh, J.; Lee, S. H.; Zoh, K. D. *J. Am. Chem. Soc.* **1992**, *114*, 7916.
(30) Suh, J.; Hah, S. S. *J. Am. Chem. Soc.* **1998**, *120*, 10 088−10 093.
(31) Suh, J.; Oh, S. *J. Org. Chem.* **2000**, *65*, 7534−7540.
(32) Oh, S.; Chang, W.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1469−1472.
(33) Kim, H.; Paik, H.; Kim, M.-s.; Chung, Y.-S.; Suh, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2557−2560.
(34) Kim, H.; Kim, M.-s.; Paik, H.; Chung, Y.-S.; Hong, I. S.; Suh, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3247−3250.
(35) Tahirov, T. H.; Oki, H.; Tsukihara, T.; Ogasahara, K.; Yutani, K.; Ogata, K.; Izu, Y.; Tsunasawa, S.; Kato, K. *J. Mol. Biol.* **1998**, *284*, 101−124.
(36) Paul-Soto, R.; Bauer, R.; Frére, J.-M.; Galleni, M.; Meyer-Klaucke, W.; Nolting, H.; Rossolini, G. M.; de Seny, D.; Hernandez-Valladares, M.; Zeppezauer, M.; Adolph, H.-W. *J. Biol. Chem.* **1999**, *274*, 13 242−13 249.
(37) Mock, W. L.; Liu, Y. *J. Biol. Chem.* **1995**, *270*, 18 437−18 446.
(38) Lippard, S. J. *Science* **1995**, *268*, 996−997.
(39) Carvajal, N.; López, V.; Salas, M.; Uribe, E.; Herrera, P.; Cerpa, J. *Biochem. Biophys. Res. Comm.* **1999**, *258*, 808−811.
(40) Williams, N. H.; Takasaki, B.; Wall, M.; Chin, J. *Acc. Chem. Res.* **1999**, *32*, 485−493.
(41) Finn, G.; Sharpless, K. B. *J. Am. Chem. Soc.* **1991**, *113*, 113−126.
(42) Shibasaki, M.; Sasai, J.; Arai, T. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1236−1256.
(43) Corey, E. J.; Bakshi, R. K.; Shibata, S. *J. Am. Chem. Soc.* **1987**, *109*, 5551−5553.

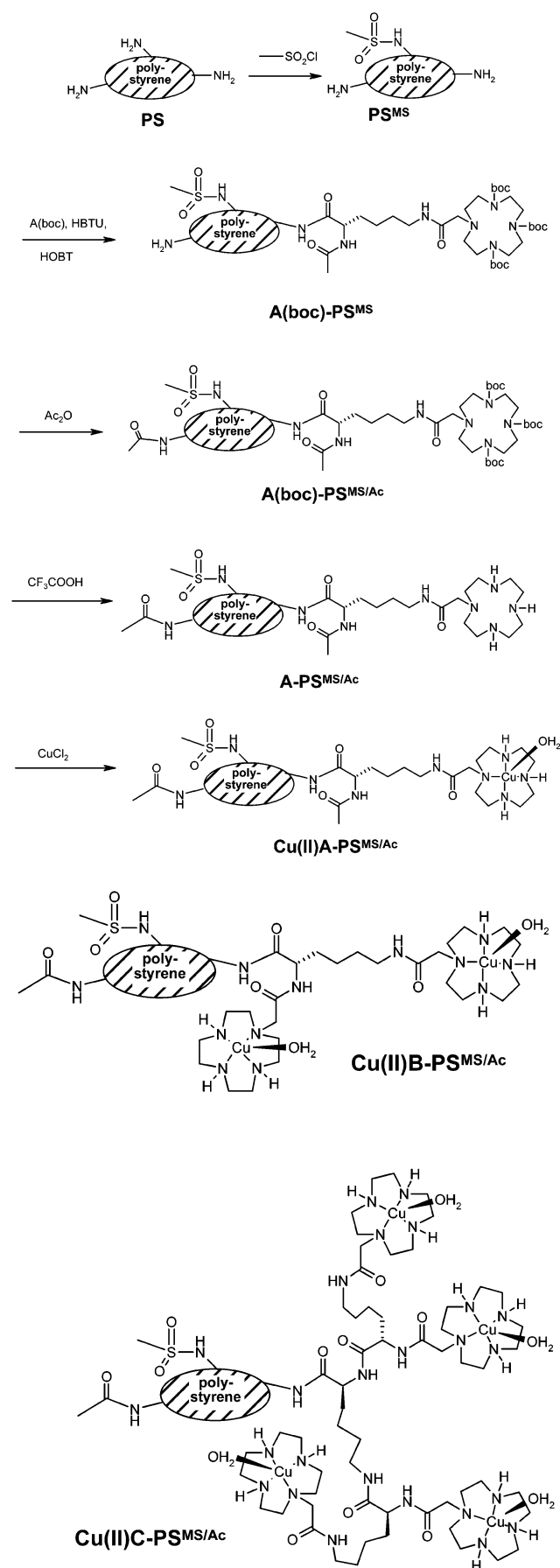(44) Izatt, R. M.; Pawlak, K.; Bradshaw, J. S. *Chem. Rev.* **1991**, *91*, 1721−2085.

moieties protected with *tert*-butyloxycarbonyl (boc) group to L-lysine (Lys). In A(boc), (boc)$_3$Cyc is attached to the ϵ-amino group of *N*-Ac-Lys. In B(boc), two (boc)$_3$Cyc moieties are attached to the α-and ϵ-amino groups of Lys. In C(boc), four (boc)$_3$Cyc moieties are attached to the amino groups of a trimer of Lys obtained by attaching two Lys moieties to the α-and ϵ-amino groups of Lys. By removing the boc protecting groups, B and C were obtained. B and C were converted in situ to Cu-(II)B and Cu(II)C, where the Cyc moieties were transformed to the Cu(II)Cyc moieties, by treating with Cu(II) ion in buffer solutions.

A(boc): X = boc

B: X = H
B(boc): X boc

C: X = H
C(boc): X = boc
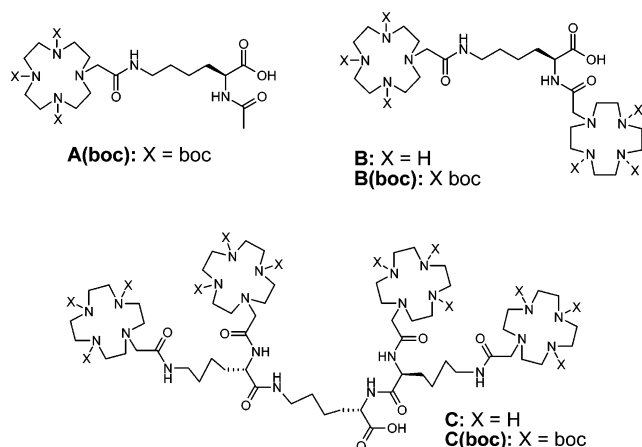
As the polymeric support of the artificial enzymes, a cross-linked polystyrene derivative (PS) containing aminomethyl groups attached to a part of the phenyl rings was used. The content of aminomethyl group in PS was 1.6 mmol/g: 17% of the phenyl rings of PS contained aminomethyl group. As summarized in Scheme 1 which illustrates the synthetic routes using Cu(II)A−PS$^{MS/Ac}$ as the example, a small portion of the amino groups were first converted to methanesulfonamido groups by treating with methanesulfonyl chloride to introduce sulfur to the polymer. Sulfur was introduced as a reference element for electron probe microanalysis (EPMA) of Cu(II) present on the polymer surface. Another small portion of the amino groups were subsequently coupled with A(boc), B(boc), or C(boc), and the rest of the amino groups were acetylated. The boc groups of Cyc moieties attached to the polymer were removed to form A−PS$^{MS/Ac}$, B−PS$^{MS/Ac}$, or C−PS$^{MS/Ac}$ with trifluoroacetic acid (TFA), which is known to quantitatively remove[45] boc groups attached to amino groups on polystyrene derivatives. By treating A−PS$^{MS/Ac}$, B−PS$^{MS/Ac}$, or C−PS$^{MS/Ac}$ with a CuCl$_2$ solution, the corresponding Cu(II) complex (Cu-(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, or Cu(II)C−PS$^{MS/Ac}$), in which Cyc moieties were converted to Cu(II)Cyc moieties, was obtained.

The contents of Cu(II) ion in Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$ were measured by inductively coupled plasma absorption-emission spectroscopy (ICP-AES) after Cu(II) ion was released from the PS derivatives by multiple treatment with 1 N HNO$_3$. On the basis of the Cu(II) content, contents of the catalytic modules were calculated as 1.9 mol % (relative to styrene moieties) for Cu(II)A−PS$^{MS/Ac}$, 1.4 mol % for Cu(II)B−PS$^{MS/Ac}$, and 1.4 mol % for Cu(II)C−PS$^{MS/Ac}$.

(45) Merrifield, B. In *Nobel Lectures in Chemistry (1981−1990)*; Frängsmyr, T. Ed.; World Scientific: Singapore, 1992; pp 143−175.

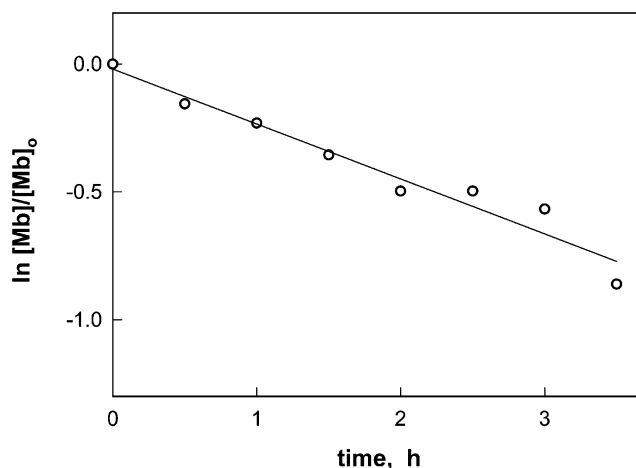**Scheme 1**. Synthetic Route to Cu(II)A−PS$^{MS/Ac}$
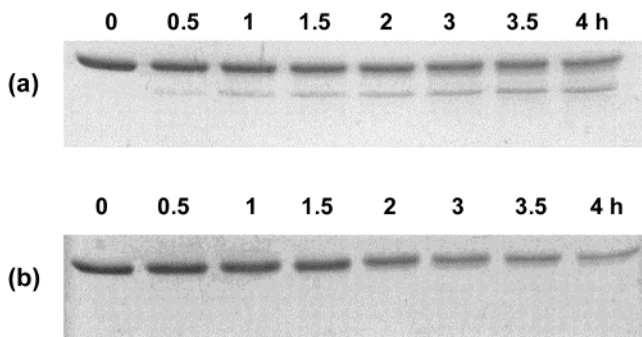
Figure 1. Results of SDS-PAGE performed on Mb incubated with the PS-based catalysts: (a) Cu(II)B−PS$^{MS/Ac}$ ($C_o = 2.3 \times 10^{-3}$ M) at pH 9.0 and 50 °C, (b) Cu(II)C−PS$^{MS/Ac}$ ($C_o = 1.1 \times 10^{-3}$ M) at pH 9.0 and 37 °C.
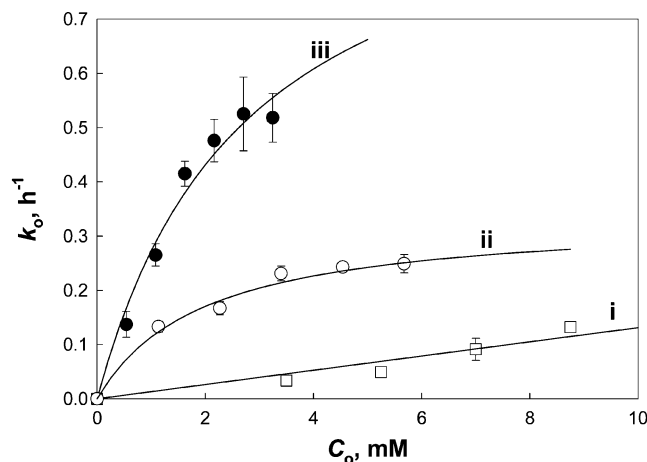
EPMA indicated that the molar ratios of Cu(II) and S on the surfaces of Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$ were 1.15, 1.27, and 2.50, respectively, which agreed well with the ratio of 1.19, 1.33, and 2.43, respectively, calculated from the Cu(II) contents determined by ICP-AES and the S contents measured by elemental analysis.

Cleavage of Myoglobin. Proteolytic activity of Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$ was examined by using horse heart Mb, 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 activity was observed with Mb. Mb is oxidized to metMb in the presence of oxygen. The Mb purchased from a commercial source and used in the present study was also in the met form as checked by its visible spectrum.[46] While the buffer solution containing Mb (concentration: 1.2×10^{-5} M) was shaken with the resin, disappearance of Mb was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis[47,48] (SDS-PAGE). Typical results of electrophoresis performed on Mb cleaved by the PS-based catalysts are illustrated in Figure 1. That the disappearance of the electrophoretic band of Mb was not due to the adsorption onto the resin was confirmed by measuring the total amino acid contents of the product solution separated from the resin according to the method described previously.[31]

The rate of protein cleavage was measured[22,30−34] by monitoring the decrease in the intensity of the electrophoretic bands corresponding to Mb. The rate data were collected at 50 °C for Cu(II)A−PS$^{MS/Ac}$ and Cu(II)B−PS$^{MS/Ac}$. The rate was considerably higher for Cu(II)C−PS$^{MS/Ac}$, for which the kinetic measurement was carried out at 37 °C. The pseudo-first-order kinetic constant (k_o) was estimated from the logarithmic plot as exemplified by Figure 2. The effect of speed of shaking the reaction mixture containing the PS-based catalyst and Mb on the rate data was examined, and it was found that k_o reached a plateau value at the shaking speed of 1200 rpm. Thus, kinetic data were collected at this shaking speed. The k_o values stand for cleavage of Mb molecule itself and do not provide information on further fragmentation of the initial cleavage products.

(46) Bodwell, C. E.; McClain P. E. In *The Science of Meat and Meat Products*, 2nd ed.; Price, J. F.; Schweigert, B. S. Ed.; W. H. Freeman: San Francisco, 1971; p 97.
(47) Lameli, U. K. *Nature* **1970**, *227*, 680−685.
(48) Hames, B. D. In *Gel Electrophoresis of Proteins*; Hames, B. D., Rickwood, D., Eds.; IRL Press: New York, 1990; Chapter 1.

Figure 2. Plot of ln [Mb]/[Mb]$_o$ against time for the data of Figure 1a.

Figure 3. The plot of k_o against C_o for the hydrolysis of the Mb catalyzed by the PS-based catalysts: (i) Cu(II)A−PS$^{MS/Ac}$ at pH 9.0 and 50 °C, (ii) Cu(II)B−PS$^{MS/Ac}$ at pH 9.0 and 50 °C, (iii) Cu(II)C−PS$^{MS/Ac}$ at pH 9.0 and 37 °C. For the catalysts, optimum activity was observed at pH 9.0 as illustrated in Figure 4. The theoretical lines are drawn with $k_{cat}/K_m = 13$ M^{-1}h^{-1} for (i), $k_{cat}/K_m = 170$ M^{-1}h^{-1} and $k_{cat} = 0.34$ h^{-1} for (ii), and $k_{cat}/K_m = 370$ M^{-1}h^{-1} and $k_{cat} = 1.0$ h^{-1} for (iii).
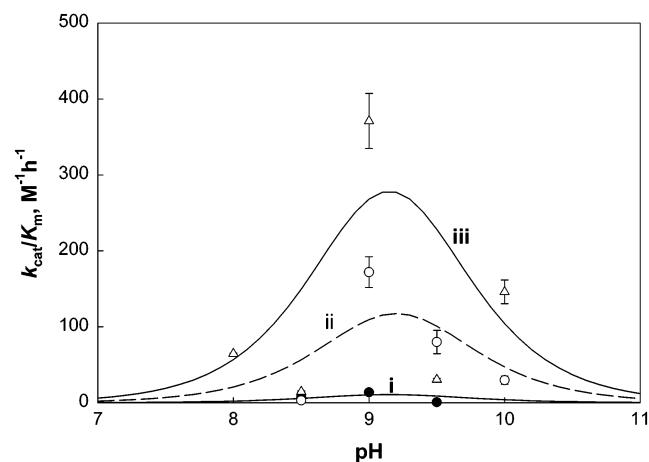
Rate data were collected by varying the amount of the catalyst (C_o). Here, C_o is expressed as the concentration of the catalytic module obtainable when the resin is assumed to be dissolved. As shown by the dependence of k_o on C_o (Figure 3), saturation kinetic behavior was observed at pH 8−10 for the cleavage of Mb by Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$.

Kinetics of the cleavage of Mb by the PS-based catalysts can be analyzed in terms of Michaelis−Menten scheme (eq 1) as was done with the reactions catalyzed by other polystyrene-based artificial enzymes.[22] Under the conditions of $C_o \gg$ [CS], pseudo-first-order kinetic behavior is expected with k_o being derived as eq 2.[22] In homogeneous systems, the quantity followed during kinetic measurement is the sum of the concentrations of free substrate ([S]) and the catalyst-substrate complex ([CS]) under the conditions of $C_o \gg$ [CS]. On the other hand, [S] is followed during the kinetic measurement in heterogeneous systems such as the reaction of the PS-based catalysts. In both cases, eq 2 is applicable. Kinetic data such as those illustrated in Figure 3 were analyzed according to eq 2 with a nonlinear regression program. For the kinetic data collected with Cu(II)A−PS$^{MS/Ac}$, k_o was proportional to C_o indicating $K_m \gg C_o$ (proportionality constant $= k_{cat}/K_m$) as

Figure 4. pH profiles of k_{cat}/K_m for cleavage of Mb by the PS-based catalysts: (i) Cu(II)A−PS$^{MS/Ac}$ at 50 °C (●), (ii) Cu(II)B−PS$^{MS/Ac}$ at 50 °C (○), (iii) Cu(II)C−PS$^{MS/Ac}$ at 37 °C (△). The theoretical lines are drawn by assuming that each catalyst is a diprotonic acid with $pK_{a1} = 9.1$ and $pK_{a2} = 9.2$ in which the monoprotonated form is reactive.

illustrated in Figure 3. The kinetic data for cleavage of Mb by Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$ were collected at various pH's. The pH dependence of k_{cat}/K_m and k_{cat} for the cleavage of Mb by the PS-based catalysts are illustrated in Figures 4 and 5.[49]

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

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

As illustrated in Figure 1, intermediate proteins accumulated in amounts detectable by the electrophoresis (SDS-PAGE) during the cleavage of Mb by Cu(II)B−PS$^{MS/Ac}$. 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 Mb was incubated with Cu(II)A−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$. MALDI-TOF MS taken for the intermediate protein mixture obtained by incubation of Mb with Cu(II)B−PS$^{MS/Ac}$ is illustrated in Figure 6. Figure 6 indicates the presence of protein fragments obtained by cleavage of Mb at two different positions: peaks with m/z of 6868 and 10 086 originate from cleavage of Mb (m/z = 16 953; the heme group dissociates during measurement of MALDI-TOF MS) at a single position and those of 6578 and 10 382 from cleavage of Mb at another position. Those intermediate proteins were further cleaved by the catalyst as shown by the MALDI-TOF MS measured after further incubation. The amino groups of the proteins and peptides present in the reaction mixtures obtained after incubation for a prolonged period were labeled with phenyl isothiocyanate and the mixture was separated by HPLC as illustrated in Figure 7.

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(49) The quality of data points in Figures 4 and 5 is not high. This has been also observed in kinetic studies with other polymeric artificial proteases.[25−27,33] The erratic data points may be ascribed to several sources: accurate measurement of densities of the parent electrophoretic band is sometimes difficult, the parent electrophoretic band may contain large degradation products in addition to the uncleaved protein substrate, properties of surface of the polymeric catalyst may be affected by pH changes which is not easily predicted, and so on. Despite scattered data points in Figures 4 and 5, the general shape of the pH profile and the optimum pH may be accurately identified and the value of a kinetic parameter measured at the optimum pH can be taken as the representative value of the catalyst.
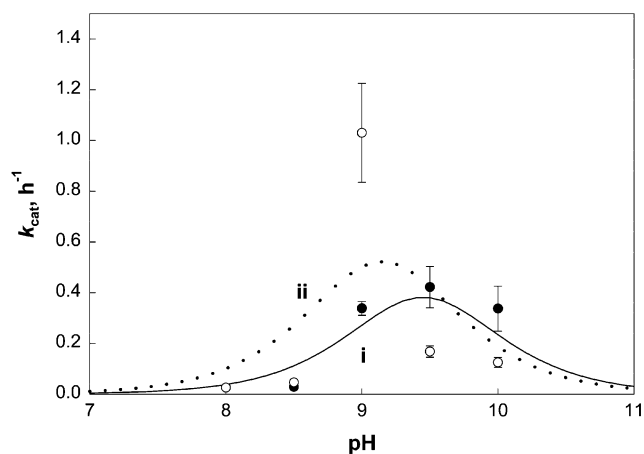
Figure 5. pH profiles of k_{cat} for cleavage of Mb by the PS-based catalysts: (i) Cu(II)B−PS$^{MS/Ac}$ at 50 °C (●), (ii) Cu(II)C−PS$^{MS/Ac}$ at 37 °C (○). The theoretical lines are drawn by assuming that each catalyst−substrate complex is a diprotonic acid ($pK_{a1} = 9.4$ and $pK_{a2} = 9.5$ for i and $pK_{a1} = 9.1$ and $pK_{a2} = 9.2$ for ii) in which the monoprotonated form is reactive.
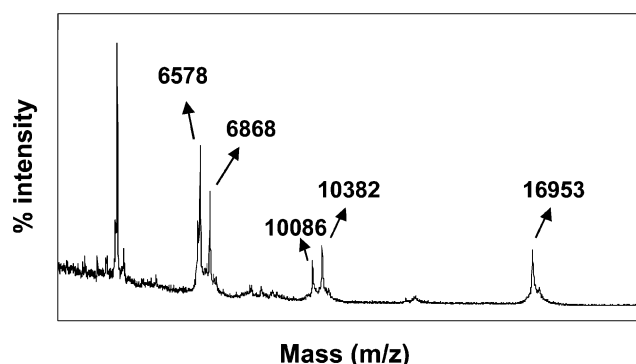

Figure 6. MALDI-TOF MS spectrum of degradation products obtained by incubation of Mb (1.2×10^{-5} M) with Cu(II)B−PS$^{MS/Ac}$ ($C_o = 2.3 \times 10^{-3}$ M) at pH 9.0 and 50 °C for 4 h.
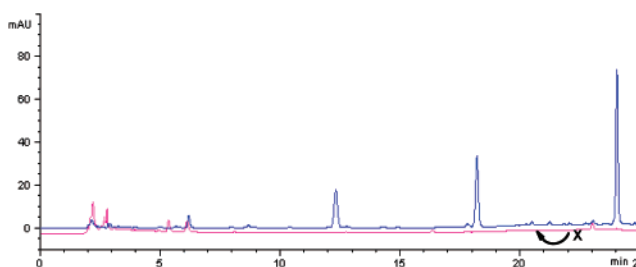
Figure 7. HPLC spectra of the product solution of a reaction mixture obtained by incubation of Mb (1.2×10^{-5} M) with Cu(II)B−PS$^{MS/Ac}$ ($C_o = 2.3 \times 10^{-3}$ M) at pH 9.0 and 50 °C for 8 h after treatment with phenyl isothiocyanate. Line x represents the signal observed without treatment with phenyl isothiocyanate. Typical conditions employed by the Waters Pico Tag System were used for the elution.

The intermediate proteins with m/z of 6868 and 10 086 may be attributed to cleavage of Mb either at Gln(91)-Ser(92), which is expected to produce two protein fragments with m/z of 6878 and 10 094, or at Leu(61)-Lys(62), which is expected to produce two protein fragments with m/z of 6909 and 10 063. On the other hand, the intermediate proteins with m/z of 6578 and 10 382 may be attributed to cleavage of Mb either at Ala(94)-Thr(95), which is expected to produce two protein fragments with m/z of 6583 and 10 389, or at Ser(58)-Glu(59), which is expected to produce two protein fragments with m/z of 6551 and 10 421.
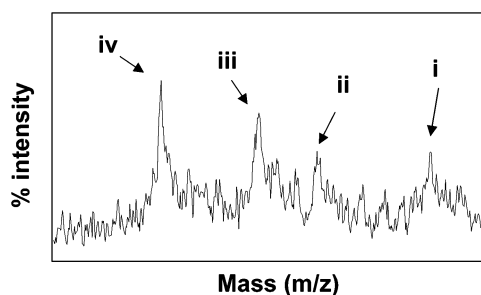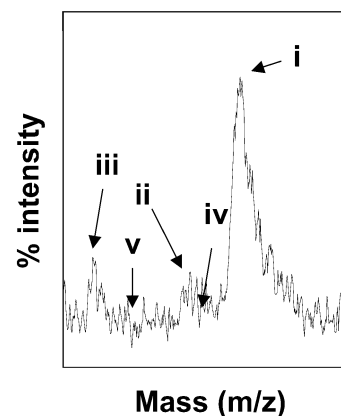
Figure 8. MALDI-TOF MS spectrum of the protein fragment with *m/z* of 10 086 (obtained as indicated in Figure 6) after treatment with CPA (1.0 × 10^{-7} M) at pH 7.5 and 25 °C for 30 m. Peak i is the protein fragment with *m/z* of 10086 and peaks ii, iii, and iv are new fragments obtained by sequential cleavage of the *C*-terminal residues with CPA. Compared with i, the *m/z* values are smaller by 131, 200, and 312 for ii, iii, and iv, respectively.

To characterize the intermediate protein fragments formed during cleavage of Mb with Cu(II)B−PS$^{MS/Ac}$, the electrophoretic band newly appearing below the band of Mb (Figure 1a) was excised from SDS-PAGE gels, destained, and extracted with the solution consisting of formic acid, water, and 2-propanol (1:3:2 v/v/v). The extract was purified and concentrated with a Nanosep centrifugal device (cutoff MW 3000). *N*-Terminal sequencing of the extracted protein mixture was carried out by Edman degradation. Amino acid residues found for each position were Gly and either Ser or Thr for the first position, Leu and His for the second position, and Ser and Ala for the third position. The results are consistent with the presence of two kinds of *N*-terminal residues: Gly(1)-Leu(2)-Ser(3) and Ser-(92)-His(93)-Ala(94). This indicates that MALDI-TOF MS peaks with *m/z* of 6868 and 10 086 originate from cleavage at Gln(91)-Ser(92). If all of the four protein fragments corresponding to MALDI-TOF MS peaks with *m/z* of 6578, 6868, 10 086, and 10 382 had been extracted from the SDS-PAGE gel, then three kinds of *N*-terminal sequences would have been observed. The failure to observe the third sequence may be ascribed to unsuccessful extraction of the protein fragment containing the third sequence.

To identify the *C*-terminal residues of the intermediate proteins, the reaction mixture separated from the catalyst was treated with carboxypeptidase A (CPA). Because CPA cleaves the *C*-terminal amino acid residues of polypeptides, MALDI-TOF MS of the cleavage product can give information on the *C*-terminal residues. Cleavage of the protein with *m/z* of 10 086 with CPA produced three additional MALDI-TOF MS peaks as illustrated in Figure 8. The decrease in the *m/z* value (131, 200, and 312 for peaks ii, iii, and iv, respectively) was consistent with the initial cleavage of Mb at Gln(91)-Ser(92) for the formation of the protein fragment with *m/z* of 10 086 (expected reduction in *m/z*: 128, 199, and 312). If the initial cleavage site is Leu(61)-Lys(62), the reduction in *m/z* is expected to be 113, 228, and 357 for ii, iii, and iv, respectively. On the basis of the results of the *N*-terminal and the *C*-terminal sequencing experiments, one of the two initial cleavage sites of Mb for the action of Cu(II)B−PS$^{MS/Ac}$ is identified as Gln(91)-Ser(92).

The protein fragment with *m/z* of 10 382 was more difficult to cleave with CPA, as revealed by the MALDI-TOF MS illustrated in Figure 9. If the initial cleavage site is Ala(94)-Thr(95), treatment with CPA is expected to produce new protein fragments corresponding to peaks ii and iii, which are stronger

Figure 9. MALDI-TOF MS spectrum of the protein fragment with *m/z* of 10 382 (obtained as indicated in Figure 6) after treatment with CPA (1.0 × 10^{-7} M) at pH 7.5 and 25 °C for 40 m. Peak i is the protein fragment with *m/z* of 10 382. Peaks ii and iii represent the new fragments expected from cleavage of the first and the second *C*-terminal residues if i was obtained by cleavage at Ala(94)-Thr(95). If i was obtained by cleavage at Ser(58)-Glu(59), the treatment with CPA is expected to produce new peaks at iv and v.

than the background signals. On the other hand, if the initial cleavage is Ser(58)-Glu(59), treatment with CPA is expected to produce peaks positioned at iv and v. Thus, both the MALDI-TOF MS of the initial protein fragments illustrated in Figure 6 and the that of the CPA-cleavage products illustrated in Figure 9 favor Ala(94)-Thr(95) rather than Ser(58)-Glu(59) as the cleavage site leading to the protein fragments with *m/z* of 6578 and 10 382. The protein fragments with *m/z* of 6578 and 6868 of Figure 6 resisted cleavage by CPA, and it was not possible to obtain any meaningful information for the nature of their *C*-terminal residues.[50] In Figure 10, the location of the proposed cleavage sites is indicated on the structure[52] of horse heart metMb obtained by X-ray crystallography.

The *N*-terminal sequencing of the intermediate proteins was carried out by Edman degradation, the fragmentation products were labeled with phenyl isothiocyanate leading to the results of Figure 7, and the *C*-terminal sequencing of the intermediate proteins was performed with CPA. These indicate that the amino and carboxyl groups were generated after cleavage of Mb with Cu(II)B−PS$^{MS/Ac}$ and demonstrate the hydrolytic nature of the protein cleavage by Cu(II)B−PS$^{MS/Ac}$.

As control experiments, rates for the cleavage of Mb by Cu-(II)B and Cu(II)C, the Cu(II)Cyc dimer and tetramer unattached to PS, were measured. At pH 10, kinetic measurements were hampered by precipitation of the Cu(II) complexes. At pH 7−9, the concentration of the Cu(II) complexes was kept below 0.04 mM due to the limited solubility. Under these conditions, Mb cleavage was not detected for 3 days at pH 7−9 and 50 °C. If it is assumed that 10% cleavage of Mb can be detected by the electrophoretic method, the upper-limit of k_o is estimated as 1 × 10^{-3} h^{-1}.

Whether the peptide bonds of Cu(II)B or Cu(II)C as well as those of the catalytic modules in Cu(II)A−PS$^{MS/Ac}$, Cu(II)C−PS$^{MS/Ac}$, or Cu(II)B−PS$^{MS/Ac}$ were cleaved by the intramolecular

(50) Gly is the *C*-terminal residue of the protein fragments with *m/z* of 6578 and 6868 whereas Ala is that of 10 382. The low reactivity of these protein fragments toward CPA is consistent with the substrate selectivity[51] of CPA.
(51) Zeffren, E.; Hall, P. L. *The Study of Enzyme Mechanism*; Wiley: New York, 1973; pp 73, 79−86, 196.
(52) Evans, S. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *213*, 885−897.
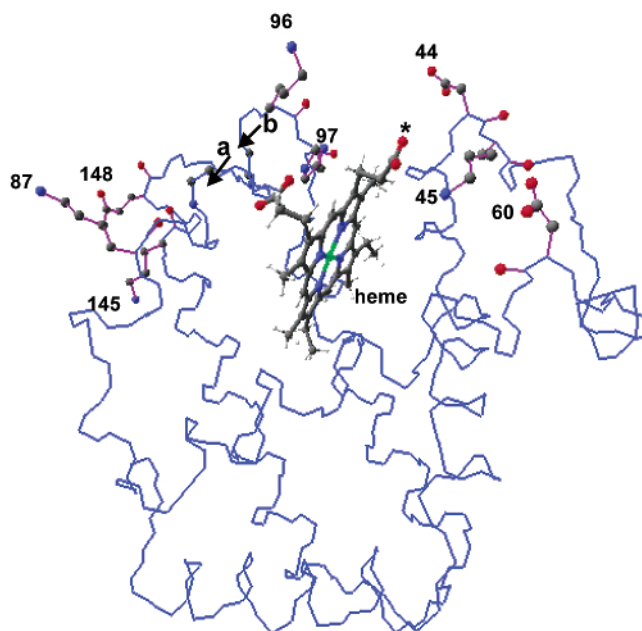
Figure 10. Location of the initial cleavage sites (a: Gln(91)-Ser(92), b: Ala(94)-Thr(95)) proposed for attack of Cu(II)B−PS$^{MS/Ac}$ at Mb and candidates for the anchorage sites on Mb for the Cu(II) center of the catalyst. The numbers indicate the side chains of the respective amino acid residues: Asp(44), Lys(45), Asp(60), Asp(87), Lys(96), His(97), Lys(145), and Glu(148). The heme carboxylate marked with * is also among the possible anchorage sites.

catalytic action of Cu(II)Cyc was examined. The catalytic activity of the PS-based catalysts for Mb cleavage and the amount of Cu(II) ion held by the resins did not change appreciably when the resins were incubated in the buffer solutions under the conditions of the kinetic measurements. Cu-(II)B and Cu(II)C were not destroyed appreciably when examined by HPLC after incubation for 3 days at pH 7−9 and 50 °C.[53]

Discussion

Catalytic modules are randomly attached to the surface of cross-linked polystyrene in Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$. If two or more catalytic modules interact with one molecule of Mb complexed on the polystyrene-based catalyst, mechanistic interpretation of the catalysis in Mb cleavage would be complicated. Mb may be considered as an oval characterized by diameters of 45, 35, and 25 Å.[54] The area occupied by each styryl monomer of the PS resin is estimated[22] as ca. 40 Å^2. Because the actual area occupied by the styryl monomer depends on its orientation on the surface of the PS resin, the average value for the effective area occupied by each monomer may be approximated as 20 Å^2. Then, about 60 styryl monomers would be accommodated in an ellipse with diameters of 45 and 35 Å. Because the catalytic modules were attached to 1.4−1.9% of the styryl monomers, the number of modules contained in the ellipse is 0.8−1.1. Even if both Mb and the PS-based catalyst were flat, therefore, only 0.8−1.1 active sites would be included in the area on the catalyst covered by one Mb molecule. Thus, it is precluded that two or more catalytic

modules of a PS-based catalyst contact the complexed Mb molecule at the same time.

Peptide bonds are quite stable with half-life for spontaneous hydrolysis being about 500−1000 years at pH 7 and 25 °C.[55,56] Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$ cleave Mb effectively by hydrolysis, manifesting saturation kinetic behavior which is analyzed by Michaelis−Menten scheme (eq 1). The k_{cat} of the Michaelis−Menten scheme represents the maximal value of the first-order rate constant achievable when substrate molecules are completely bound by the catalyst. The k_{cat} values at the optimum pH for Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$ are 0.34 h^{-1} (pH 9 and 50 °C) and 1.0 h^{-1} (pH 9 and 37 °C), respectively, corresponding to half-lives of 2 and 0.7 h. These 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[57] with peptidase activity elicited by a joint hybridoma and combinatorial antibody library approach in the hydrolysis of an amide substrate.

Figure 3 illustrates kinetic data obtained for the PS-based catalysts at their optimum pH's. The kinetic data for Cu(II)C−PS$^{MS/Ac}$ were measured at 37 °C whereas those for Cu(II)A−PS$^{MS/Ac}$ and Cu(II)B−PS$^{MS/Ac}$ 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 Cu(II)A−PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, and Cu(II)C−PS$^{MS/Ac}$ and the ratio of k_{cat} as 1:10 for Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$. 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*). As the catalytic group density of the module is increased in the PS-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 PS-based catalysts, catalytic activity was considerably improved simply by raising the catalytic group density without deliberate positioning of the catalytic groups.

Saturation kinetic behavior was not observed (Figure 3) with Cu(II)A−PS$^{MS/Ac}$, in contrast to Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$. Thus, metal centers contained in a dinuclear or tetranuclear catalytic module of Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$ appear to cooperate effectively both in reducing K_m and in raising k_{cat}/K_m.

The MALDI-TOF MS of the reaction mixture (Figure 6) revealed two pairs of protein fragments formed by the initial cleavage of Mb by Cu(II)B−PS$^{MS/Ac}$. One of the two initial cleavage sites is identified as Gln(91)-Ser(92) and the other is suggested as Ala(94)-Thr(95). High site selectivity manifested by Cu(II)B−PS$^{MS/Ac}$ may be attributed to anchorage of one Cu-(II)Cyc of the catalyst to a site on Mb and interaction of the other Cu(II)Cyc center with the scissile peptide bond. The scissile peptide bond and the anchorage site may be connected by a linker composed of several covalent bonds so that the catalytic action involves through-bond two-point interaction between the catalyst and Mb. On the other hand, the two sites may take remote positions in the primary structure but occupy proximal positions in the tertiary structure so that the catalytic

(53) Negligible hydrolysis of the peptide bonds of the catalytic modules either on the surface of PS or in the bulk water suggests that the intramolecular attack by the Cu(II)Cyc group at the peptide linkage involves steric strain.
(54) Stryer, L., Freeman, W. H., Eds.; *Biochemistry*; New York, 1995; 4th, p 149.

(55) Bryant, R. A. R.; Hansen, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 5498−5499.
(56) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105−6109.
(57) 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.

action takes place by through-space two-point interaction between the catalyst and Mb.

For the through-bond two-point interaction, a functional group present in the side chain of an amino acid residue located in vicinity to the cleavage site is to be used as the anchorage site. In the case of cleavage at Gln(91)-Ser(92), the imidazolyl group of His(93) appears to be the only metal-coordinating group located in an appropriate position among the functional groups of those amino acid residues (Pro(88), Leu(89), Ala(90), Gln-(91), Ser(92), His(93), Ala(94)).[58] With the peptide carbonyl group of Gln(91)-Ser(92) interacting with a Cu(II)Cyc moiety, the imidazole of His(93) may be bound to the other Cu(II)Cyc center. Examination with a CPK model and molecular modeling programs (HyperChem and Sculpt) suggested that the through-bond two-point interaction may produce tetrahedral intermediate D in the action of a Cu(II)B derivative on an oligopeptide containing Gln-Ser-His. This mechanism is unlikely, however, because His(93) is the proximal[59] histidine whose imidazole is coordinated to the Fe ion of the heme group of Mb.[52]


D
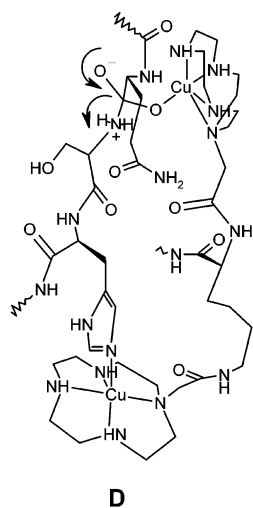
For the mechanism of the through-space two-point interaction, various functional groups of Mb may be considered as the anchorage site. For the cleavage at Gln(91)-Ser(92) or Ala(94)-Thr(95), possible anchorage sites suggested by the crystallographic structure of metMb are indicated in Figure 10. Mb contains 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 PS-based catalysts. It is not easy to rationalize why the Cu(II) is anchored by the functional groups indicated in Figure 10 instead of 40 or more other groups. In this regard, it is most reasonable to choose the heme carboxylate group as the anchorage site. The Mb 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 Cu(II)B–PS[MS/Ac] 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

(58) Histidyl imidazole is utilized as a ligand for Cu(II) ion in various Cu(II) enzymes such as ascorbate oxidase, plastocyanin, hemocyanin, and copper zinc superoxide dismutase.[59]

(59) Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, 1994; pp 87, 241, 285, 286, 300, 328.
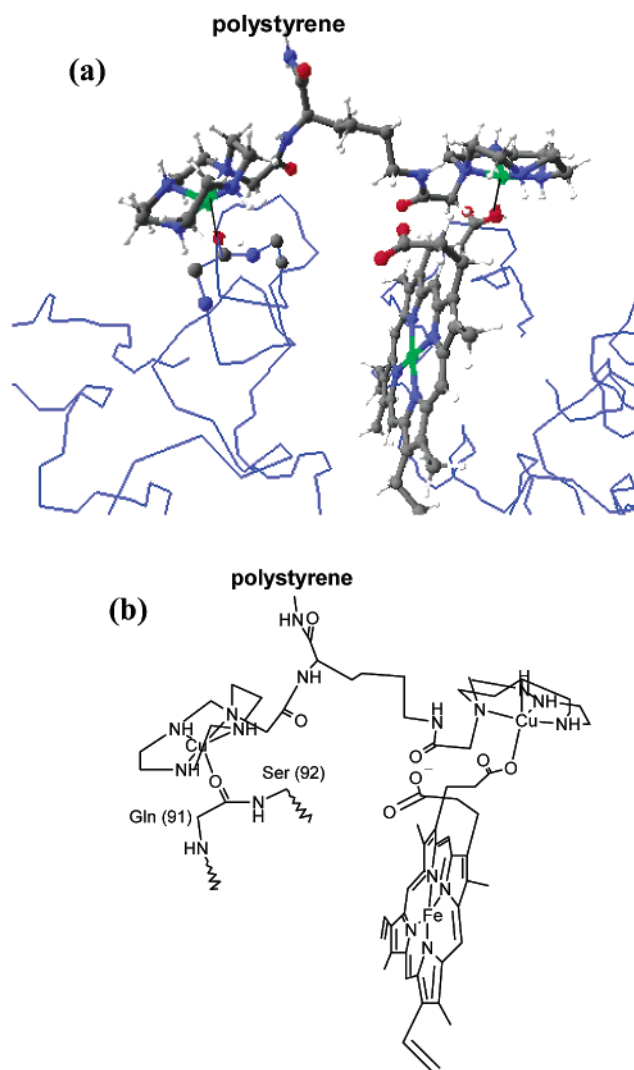

Figure 11. Structure of complex formed between Cu(II)B–PS[MS/Ac] and Mb by the through-space two-point interaction leading to the cleavage of Mb at Gln(91)-Ser(92) by the mechanism of F: (a) results of molecular modeling shown with backbone of Mb displayed in the background, (b) schematic diagram for the interaction. The structure was obtained through optimization of the structure of the Cu(II)B portion by molecular modeling programs with the structure of Mb frozen as the crystalline structure.

with the size of the linker connecting the two Cyc moieties. As illustrated in Figure 11, interaction of the catalyst with Mb at Gln(91)-Ser(92) does not involve steric interaction with other parts of the protein when examined with molecular modeling programs (HyperChem and Sculpt). A similar situation is predicted for the cleavage at Ala(94)-Thr(95) by the modeling programs. Examination of the crystallographic structure of Mb for the mechanistic analysis ignores the dynamic nature of the complex formed between the Mb and the catalyst. Crystallographic structures of many enzymes, however, have been used to deduce mechanistic information despite the static nature of the crystal structures. By analogy, analysis summarized in Figure 11 may lead to useful mechanistic implications.

Either attack (E) by the Cu(II)-bound hydroxo ion at the carbonyl carbon or attack (F) by an external hydroxide ion at the carbonyl group bound to the Cu(II) center is possible leading to the formation of the tetrahedral intermediate whose breakdown is rate-determining (G).[60,61] Mechanisms E and F are not easily differentiated. It has been shown that both the attack by

the metal-bound hydroxide and attack by external hydroxide ion take place for the Co(III)-catalyzed hydrolysis of peptide bonds.[10]

E

F

G
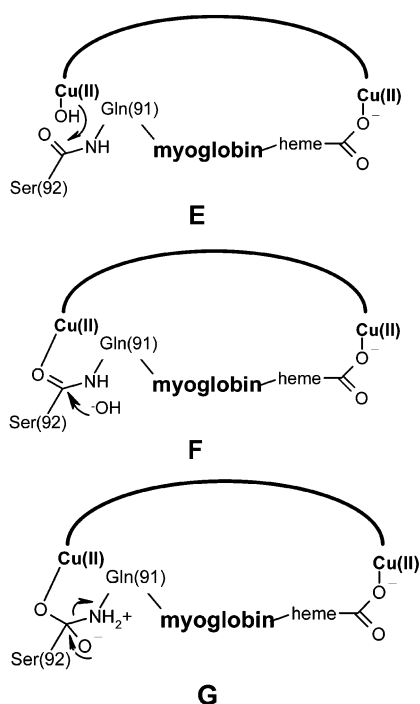
Considerably higher activity was manifested by Cu(II)C−PS$^{MS/Ac}$ compared with Cu(II)B−PS$^{MS/Ac}$ in terms of both k_{cat} and k_{cat}/K_m. No intermediate proteins accumulated in an amount detectable by electrophoresis or MALDI-TOF MS during Mb cleavage by Cu(II)C−PS$^{MS/Ac}$ in contrast to that by Cu(II)B−PS$^{MS/Ac}$. Both the higher activity and lack of accumulating intermediate may be attributed to the presence of extra Cu(II)-Cyc centers that can attack at the complexed Mb molecule. If one of the four Cu(II)Cyc centers of the tetranuclear catalytic module of Cu(II)C−PS$^{MS/Ac}$ is held by the anchorage site, then the remaining three Cu(II)Cyc centers may be able to attack at several peptide bonds of the complexed Mb, producing several protein fragments. The tetranuclear catalytic module may be also more effective than the dinuclear catalytic module in degradation of the initial cleavage products to smaller fragments. In the degradation of the cleavage products by Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$, functional groups such as carboxylate, imidazolyl, phenolate, amino, or methylthio groups provided by the peptide backbone of the cleavage products may be utilized by the Cu(II)Cyc-containing catalysts as the anchorage sites.

By analogy with enzyme kinetics,[51] the pH profile of k_{cat}/K_m can be interpreted in terms of ionization of functional groups in *C* and *S* and the pH profile of k_{cat} in terms of that in *CS*.[49] In

the mechanism of E or F, one of the Cu(II) centers of the catalyst should have an aquo ligand to accommodate complex formation between Mb and the catalyst. Moreover, either a hydroxo ion bound to the other Cu(II) center of the catalyst (mechanism E) or an external hydroxide ion (mechanism F) is needed for cleavage of the scissile peptide bond. The bell-shaped pH profiles for k_{cat}/K_m (Figure 4) is consistent with this analysis. The bell-shaped pH profiles for k_{cat} (Figure 5) may be explained in terms of the mechanism of G because protonation of the alkoxide anion at low pH and deprotonation of the ammonium cation at high pH would reduce k_{cat}. In addition, replacement of either alkoxide or carboxylate anion coordinated to Cu(II) in the mechanism of G with hydroxide ion at high pH's would also inhibit the reaction.

For the PS-based catalytic modules, C_o is the concentration attainable when the resin is dissolved in solution. Only a very small fraction of the catalytic modules are exposed to the open area that can accommodate Mb molecule. Although C_o employed for Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$ is up to 6 mM (Figure 2), the actual concentration of the catalytic module available for attack at Mb would not be significantly greater than C_o (40 μM) used for Cu(II)B or Cu(II)C dissolved in water. Comparison of the rate data obtained for Cu(II)B or Cu(II)C with Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$ reveals that the proteolytic activity of the dinuclear or tetranuclear catalytic module is enhanced considerably on attachment to the polystyrene resin. This may be related to the microenvironments provided by polystyrene. Previous studies indicated that the intrinsic reactivity of metal complexes of Cyc in hydrolysis of proteins or double-stranded DNA is considerably enhanced when the metal centers are attached to polystyrene.[22,26,27] In addition, the binding of carboxylate anion of the heme of Mb by the Cu-(II) center of the catalyst would be considerably stronger in the hydrophobic domain of the polystyrene than in bulk water.[24,25]

Little proteolytic activity was manifested by Cu(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$ toward γ-globulin or albumin, whereas selectivity in cleavage site was demonstrated by Cu(II)B−PS$^{MS/Ac}$ toward Mb. The proteins obtained as intermediates by cleavage of Mb were, however, effectively cleaved by the polymeric artificial proteases. For effective degradation of a protein substrate by a polymeric catalyst, it is prerequisite that the substrate is complexed effectively to the surface of polymer support. It appears that several factors such as functional groups covering the surface of polymer support and polarity of the microenvironments on the polymer support as well as conformation and surface properties of protein substrates play important roles in the complexation of proteins.[62] It appears that proteins produced by initial cleavage of Mb are readily complexed to the polymeric catalysts examined in the present study whereas albumin and γ-globulin are not. How to facilitate[63] complex formation between a polymeric artificial protease and various protein substrates and, at the same time, to achieve selectivity in cleavage site is the next target in the

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(60) Presence of the intermediate of G, which requires at least two Cu(II)Cyc moieties, is consistent with the much smaller K_m values observed with Cu-(II)B−PS$^{MS/Ac}$ or Cu(II)C−PS$^{MS/Ac}$ compared with Cu(II)A−PS$^{MS/Ac}$.[51]

(61) To check whether small oligopeptides can be hydrolyzed by the polystyrene-based catalysts by analogy with the mechanism of G, *trans*-cinnamoyl-L-Phe-Gly-Gly-L-Phe was tested as a substrate. HPLC analysis of the reaction mixture obtained after incubation of the oligopeptide with Cu(II)A-PS$^{MS/Ac}$, Cu(II)B−PS$^{MS/Ac}$, or Cu(II)B−PS$^{MS/Ac}$ at pH 9.0 and 37 °C for 1 d (C_o= 1.1 × 10^{-3} M, S_o =1.8 × 10^{-4} M) revealed, however, neither the oligopeptide nor its hydrolysis products containing the cinnamoyl chromophore were detected. This indicates ready adsorption of the oligopeptide substrate and/or its hydrolysis products onto the resins.

(62) For example, another derivative of polystyrene containing Cu(II)Cyc prepared in a previous study effectively cleaved[22] γ-globulin. This catalyst, however, was not effective toward albumin. On the other hand, an artificial protease prepared by construction of an artificial active site comprising three salicylate residues on polystyrene effectively cleaved albumin[32] but was not active toward γ-globulin. Similarly, an artificial protease prepared by attaching two or more proximal imidazoles on polystyrene manifested high proteolytic activity toward albumin[31] but did not cleave γ-globulin.

studies on polymeric artificial proteases aimed at practical applications in protein industry.

In conclusion, the dinuclear or tetranuclear catalytic modules attached to polystyrene manifested high proteolytic activity toward Mb due to the cooperative action of the Cu(II)Cyc units in substrate recognition and cleavage of peptide backbone of the complexed substrate. Because of selective binding of the catalyst to the anchorage site, certain peptide bonds of Mb were selectively cleaved in the action of the dinuclear artificial protease. The high site selectivity for the initial cleavage of Mb and mechanistic analysis of the catalytic action are unprecedented for polymeric artificial enzymes.

Experimental Section

Preparation of Catalysts. The derivative (PS) of poly(styrene-*co*-divinylbenzne) with 17% of styryl residues aminomethylated (1.6 mmol NH$_2$ per gram resin) and with 2% cross-linkage was purchased from Fluka. To a suspension of PS (2.0 g; 15 residue mmol of styrene monomer) in 20 mL of methylene chloride (MC), methanesulfonyl chloride (0.035 mL; 0.45 mmol) and triethylamine (TEA) (0.46 mL; 3.2 mmol) were added and the mixture was shaken at 45 rpm and room temperature for 1 day. The methanesulfonyl-containing resin (PS^{MS}) was collected by filtration, washed with MC (30 mL × 5), MeOH (30 mL × 5), and dried in vacuo. Two batches of PS^{MS} were prepared: one (sulfur content: 2.1 mol % of styryl moieties as measured by elemental analysis) was used for preparation of Cu(II)A−PS$^{MS/Ac}$ and the other (sulfur content: 1.6 mol %) for that of Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$. A solution (5 mL) of A(boc) (310 mg; 0.44 mmol) mixed with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (180 mg; 0.47 mmol), di-*i*-propylethylamine (DIEA) (0.17 mL; 0.98 mmol), and *N*-hydroxybenzotriazole (HOBT) (66 mg; 0.49 mmol) in 5 mL *N*,*N*-dimethylformamide (DMF) was added to the suspension of PS^{MS} (2 g) in 20 mL of DMF. The suspension was degassed for 30 m and shaken at 45 rpm and room temperature for 1 day. The product resin (A(boc)−PS^{MS}) was collected by filtration, washed with DMF (30 mL × 5), MC (30 mL × 5), MeOH (30 mL × 5), and dried in vacuo. The unreacted amino groups of A(boc)−PS^{MS} were acetylated by shaking the resin (2 g) with acetic anhydride (2.8 mL) and TEA (5.2 mL) in 20 mL DMF at room temperature for 1 day. The product resin (A(boc)−PS$^{MS/Ac}$) was collected by filtration, washed with DMF (30 mL × 5), MeOH (30 mL × 5), and dried in vacuo. Kaiser test[65] indicated that the yield of the acetylation step was greater than 99%. A(boc)−PS$^{MS/Ac}$ (2 g) was shaken in the mixture of 5 mL TFA and 15 mL MC at 45 rpm and room temperature for 1 h to remove the boc groups. The product resin (A−PS$^{MS/Ac}$) was collected by filtration, washed with MC (30 mL × 5), 10% DIEA in MC (20 mL

× 2), and MeOH (30 mL × 5), and dried in vacuo. To 1.0 M CuCl$_2$·2H$_2$O solution in DMF (20 mL), A-PS$^{MS/Ac}$ (1.5 g) was suspended and the resulting mixture was shaken at 45 rpm and room temperature for 1 day. The product resin (Cu(II)A−PS$^{MS/Ac}$) was collected by filtration, washed with DMF (30 mL × 5) and MeOH (30 mL × 5). Then, Cu-(II)A−PS$^{MS/Ac}$ was resuspended in the pH 6 buffer and the mixture was shaken at 45 rpm and room temperature for 1 day. Cu(II)A−PS$^{MS/Ac}$ was collected by filtration, washed with water (30 mL × 5) and MeOH (30 mL × 5) and dried in vacuo. B(boc) and C(boc) were attached to PS^{MS} by the method described above for the preparation of A(boc)−PS^{MS}. The resulting resins were treated with acetic anhydride, TFA, and CuCl$_2$, in sequence, as described above for the preparation of Cu-(II)A−PS$^{MS/Ac}$ to obtain Cu(II)B−PS$^{MS/Ac}$ and Cu(II)C−PS$^{MS/Ac}$. As mentioned above, contents of the catalytic modules are 1.9 mol % (relative to styrene moieties) for Cu(II)A−PS$^{MS/Ac}$, 1.4 mol % for Cu-(II)B−PS$^{MS/Ac}$, and 1.4 mol % for Cu(II)C−PS$^{MS/Ac}$. These correspond to 79%, 60%, and 58% yields for the coupling steps of PS^{MS} with A(boc), B(boc), and C(boc), respectively.

Measurements. In kinetic measurements, the shaking speed and temperature were controlled with a VORTEMP manufactured by Labnet. pH measurements were carried out with a Dongwoo Medical DP-880 pH/Ion meter. The degree of cleavage of proteins was measured by SDS-PAGE with a Mighty Small II SE 250 model. Densities of the electrophoretic bands were analyzed with a AlphaImager 2200 model and a AlphaEase model. MALDI-TOF MS analysis was performed with a Voyger-DE STR Biospectrometry Workstation Model. UV−Vis spectra were taken with a Beckman DU 68 spectrophotometer. *N*-terminal sequencing was carried out by Korea Basic Science Research Institute with a Procise 491 protein sequencer. ICP-AES measurement was carried out with a Shimadzu ICPS-1000IV model. EPMA was performed with a CAMECA SX-57 model. Distilled and deionized water was used for preparation of buffer solutions. Buffers (0.05 M) used in this study were *N*-2-hydroxyethylpiperazine-*N*′-ethansulfonate (pH 7−8), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (pH 8.5) and boric acid (pH 9−10). All buffer solutions were filtered with 0.45 μm Millipore microfilter and autoclaved before use in the kinetic measurements. The stock solution of Mb ([Mb] = 1.2 × 10^{-4} M) was prepared by dissolving horse heart Mb (purchased from Sigma, used without further purification) in water and was kept at 4 °C. Bovine CPA, albumin, and γ-globulin were purchased from Sigma and used without further purification. The resins were suspended in a buffer solution and swollen for 1 h prior to kinetic measurement. After Mb (final concentration: 1.2 × 10^{-5} M) was added, the resulting mixture was shaken at the speed of 1200 rpm and at 37 °C or 50 °C. The mixture was centrifuged at various intervals and aliquots (20 μL) of the supernatant were collected for analysis by SDS-PAGE.

Supporting Information Available: Experimental procedures for synthesis of A(boc), B(boc), B, C(boc), C, and *trans*-cinnamoyl-L-Phe-Gly-Gly-L-Phe. This material is available free of charge via the Internet at http:/pubs.acs.org.

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(63) If the protein substrates or their hydrolysis products are bound too strongly and, thus, adsorbed onto the polymeric support, then the polymer has little catalytic value. For example, γ-globulin and albumin are strongly adsorbed onto the Amberlite weakly acidic cation exchanger in which polystyrene is the matrix and carboxyl group is the active group.[64]

(64) Kim, H.; Chung, Y.-S.; Paik, H.; Kim, M.-s.; Suh, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2663−2666.

(65) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.