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Functional Molecules Based on Polyazometals. (1) Artificial Metalloproteinases Prepared by Conjugation of Polyazometals with Poly(allylamine)

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Abstract: Polyazometals (PAMs) conjugated with poly(allylamine) manifested high catalytic activity in the hydrolytic cleavage of bovine serum albumin. Based on cleavage sites, the mechanism for the PAM-catalyzed peptide hydrolysis is suggested.

As a novel methodology for designing the active sites of artificial enzymes, we have developed self-assembly of metal ions and a ligand equipped with two chelating sites.[1] The self-assembly can lead to coordination polymers or molecular clusters containing multiple number of metal centers positioned in proximity. Metal ions have been utilized as catalytic groups in many biomimetic catalysts since they are far more versatile than organic functional groups in catalyzing organic reactions.[2] Participation of multiple metal centers may lead to highly effective catalysis as observed with many metalloenzymes. In this letter, we report high proteinase activity of catalysts obtained by conjugation of the self-assembly of metal centers to poly(allylamine) (PAA).

In previous studies,[3] *o,o'*-dihydroxyazobenzene (DHAB) mixed with Fe(II) and Fe(III) ions was found to form solid materials with intrinsic conductivity. To explain the intrinsic conductivity, formation of coordination polymers (polyazometal; PAM) such as **1** was proposed. In addition, sonication of amphiphile **2** and its analogue in the presence of transition metal ions produced coordinatively polymerized bilayer membranes (CPBMs) as schematically illustrated by **3**.[4] Upon complexation with the transition metal ions, stability of the bilayer membranes was remarkably improved. Amphiphile **2** forms complexes with transition metal ions with 1:1 stoichiometry. The pronounced enhancement of the stability of bilayer membranes suggests that each metal ion of the resulting CPBM is bound to two nitrogen atoms of two adjacent DHAB moieties as indicated by **1** and **3**. Some metal ions may be attached to one DHAB unit in **3**. Even in that case, a polymeric cluster of metal ions bound to the DHAB ligand is obtained upon treatments of **2** with transition metal ions.[5] The CPBMs of **2**

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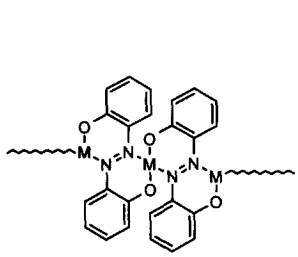
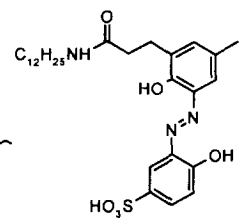
obtained in the presence of Co(III) or Fe(III) ion were found to cleave chymotrypsin and carboxypeptidase A by hydrolysis.[1] Inactivation of ChT by multiple cleavage was complete within a few minutes at 4 °C and pH 7.5 when the amphiphile concentration was 5.12 mM.[1] This corresponds to more than 10^9-fold acceleration when compared with the rates for spontaneous hydrolysis[6] of peptide bonds.



1



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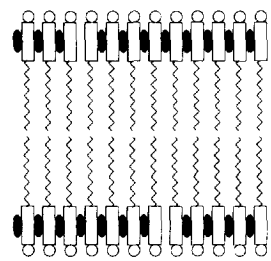
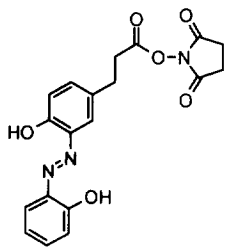


3

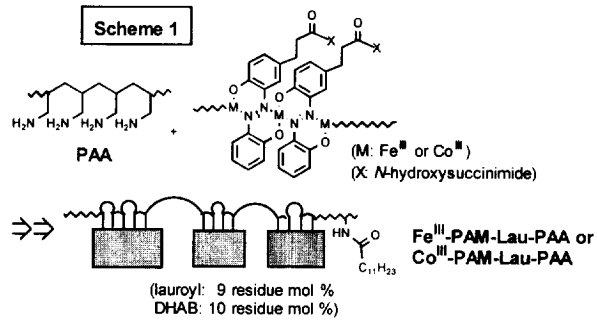
Although the CPBMs manifested remarkable catalysis in peptide hydrolysis, it showed activity only toward chymotrypsin and carboxypeptidase A among about 10 proteins examined. Since chymotrypsin and carboxypeptidase A are proteases, the possibility of acceleration of self digestion in the presence of the CPBMs was not positively excluded. In addition, the bilayer membranes have inherent limitations due to instability in organic solvents. Moreover, phase transitions at various temperatures complicate examination of the catalytic activity of CPBMs at various temperatures.

In attempts to design a new type of PAM derivatives that can overcome these problems, we have prepared PAM conjugated with PAA. Compound **4** (mp 204-207°C) was prepared by coupling 2,2'-dihydroxy-5-carboxyethylazobenzene with *N*-hydroxysuccinimide in the presence of an equivalent of *N,N'*-dicyclohexylcarbodiimide and a catalytic amount of *N,N*-dimethylaminopyridine in a 20:1 mixture of chloroform and DMF at room temperature followed by recrystallization from ethyl acetate. In DMSO, **4** is bound by Fe(III) ion strongly to form a 1:1-type complex as checked by a spectrophotometric method[7] reported previously. A DMSO solution of PAA (average degree of polymerization = ca. 600; the HCl salt purchased from Aldrich was treated with an equivalent of NaOH in ethanol) was added to a DMSO solution of PAM obtained by mixing **4** with an equivalent of $FeCl_3$ or $[Co(NH_3)_5Cl]Cl_2$. Acylation of the amino groups of PAA with the activated ester linkages of PAM produced a PAM-PAA conjugate. Amino groups of the resulting polymer were lauroylated with the *N*-hydroxysuccinimide ester (mp 75-76°C) of lauric acid in DMSO leading to the formation of Fe^{III}-PAM-Lau-PAA or Co^{III}-PAM-Lau-PAA as summarized in Scheme 1. The PAM-PAA conjugates were purified by repetitive dialysis both before and after lauroylation.



4

Scheme 1



By the reaction of **4** with the lauroylated PAA[8] in DMSO in the absence of any added transition metal ions, the DHAB moieties were attached randomly to PAA backbone to obtain DHAB-Lau-PAA which was purified by repetitive dialysis. By mixing an aqueous solution of $FeCl_3$ or $[Co(NH_3)_5Cl]Cl_2$ with DHAB-Lau-PAA, Fe^{III}-DHAB-Lau-PAA or Co^{III}-DHAB-Lau-PAA, respectively, was obtained.

The shaded rectangles in Scheme 1 stand for PAM regions. Although the sizes of the PAM regions are unknown at present, the amount of **4** added during the preparation of the PAM-PAA conjugates and DHAB-Lau-PAA was 10 residue mol % relative to PAA. That the attachment of DHAB to PAA proceeded almost qauntitatively was confirmed by the absence of any appreciable amount of azo molecules leaching out from the polymers during dialysis. The amount of the lauroylating ester added during the preparation of the PAM-Lau-PAA conjugates or DHAB-Lau-PAA was 10 residue mol % relative to PAA. Elemental analysis of the product indicated that 80-90 % of the lauroylating ester was attached to PAA.

In Fig. 1, the progress of cleavage of bovine serum albumin (BSA) (M.W. 66000) by Co^{III}-PAM-Lau-PAA is illustrated with the reaction mixtures analyzed by electrophoresis (SDS-PAGE[9]). The time indicated in Fig. 1 represents the period of incubation. The concentration of the polymer is expressed in terms of the amount of the DHAB monomer in the present study. Fig. 1 reveals that the parent band of BSA diminishes continuously and that several intermediate proteins accumulate and break down when BSA is incubated with Co^{III}-PAM-Lau-PAA. For the cleavage of BSA by Fe^{III}-PAM-Lau-PAA, on the other hand, accumulation of intermediate proteins was not observed, indicating faster breakdown of the intermediates compared with the corresponding formation step. When the parent band for BSA disappeared completely, no bands were observed by electrophoresis. The mixture (74 % recovered by ultrafiltration with a centrifugal concentrator: cutoff M.W. of 30000) of peptides obtained as the product for the cleavage of BSA by Fe^{III}-PAM-Lau-PAA was subjected to *N*-terminal sequencing by Edman degradation. This allowed estimation of the amount of amino acid residues that occupied the *N*-terminal positions. The results disclosed that ca. 3 % of amino acids initially contained in BSA became the *N*-terminal residues of the product peptides. The kinetic data for BSA cleavage were not affected by exclusion of oxygen or light. These results indicate that BSA is cleaved by hydrolysis of the peptide bonds.[1]

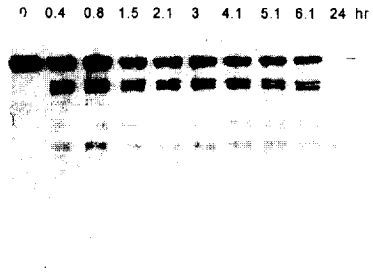


Fig. 1. Separation of proteins by electrophoresis during the cleavage of BSA (5.3 X 10^{-6} M) by Co^{III}-PAM-Lau-PAA (3.5 X 10^{-5} M) at 50 °C and pH 8.5.

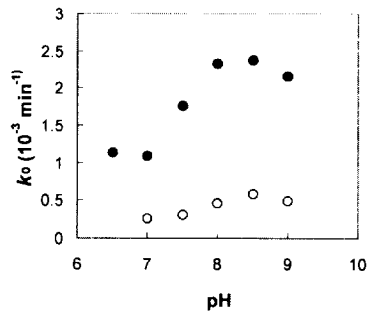


Fig. 2. pH dependence of k_o for BSA hydrolysis by Co^{III}-PAM-Lau-PAA (●) or Co^{III}-DHAB-Lau-PAA (O) at 50°C: C_o = 3.5 X 10^{-5} M, S_o = 5.1 X 10^{-6} M.

Analysis of density of the parent band observed by electrophoresis revealed that the decay followed pseudo-first-order kinetics up to at least 2 half-lives. The pseudo-first-order rate constants (k_o) for disappearance of the parent band was measured at a fixed concentration of the catalyst at various pHs and are summarized in Fig. 2 and 3. At the optimum pH, the dependence of k_o on C_o (the initially added concentration of the catalyst) was measured and is illustrated in Fig. 4 and 5. The slopes of the straight lines drawn in Fig. 4 and 5 are 63 ± 3 and 410 ± 30 $M^{-1}min^{-1}$, respectively. The data points for the highest k_o values indicated in Fig. 4 and 5 correspond to the half-lives of 140 and 40 min at 50°C, respectively. These values of half-lives can be compared with that of 500-1000 yr for the spontaneous hydrolysis of peptide bonds at 25 °C and pH 7.[6] The degree of acceleration for peptide hydrolysis achieved by Co^{III}-PAM-Lau-PAA or Fe^{III}-PAM-Lau-PAA is about 10^6-fold. The bell-shaped pH profiles of Fig. 2 and 3 stand in contrast with the pH independent rates[1] observed previously for protein hydrolysis by CPBMs. The difference is not easy to explain since shapes of the pH profiles reflect the ionization of functional groups of both the substrate and the catalyst.

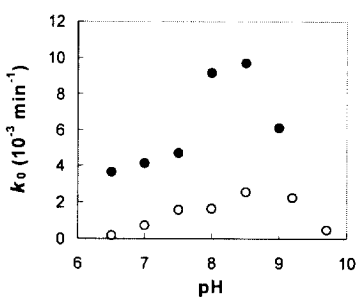


Fig. 3. pH dependence of k_o for BSA hydrolysis by Fe^{III}-PAM-Lau-PAA (●) or Fe^{III}-DHAB-Lau-PAA (O) at 50°C: C_o = 2.4 X 10^{-5} M, S_o = 5.3 X 10^{-6} M.

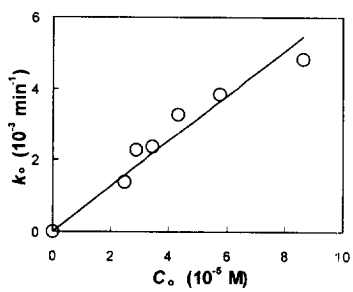


Fig. 4. Plot of k_o against C_o for Co^{III}-PAM-Lau-PAA at 50°C: S_o = 5.1 X 10^{-6} M

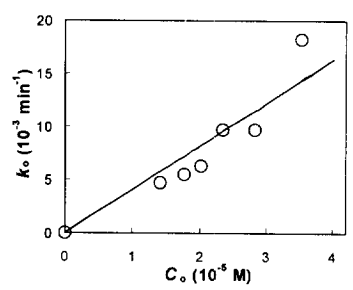


Fig. 5. Plot of k_o against C_o for Fe^{III}-PAM-Lau-PAA at 50°C: S_o = 5.3 X 10^{-6} M

When the PAM-PAA conjugates were not lauroylated, cleavage of BSA was much slower. Lauroyl groups

may facilitate complexation of BSA to the catalyst, leading to faster reactions. Cleavage of BSA by the complexes prepared by random attachment of DHAB to PAA (Fe^{III}-DHAB-Lau-PAA and Co^{III}-DHAB-Lau-PAA) was also examined. Under the identical conditions, the proteinase activity of Fe^{III}-DHAB-Lau-PAA or Co^{III}-DHAB-Lau-PAA was substantially lower than that of Fe^{III}-PAM-Lau-PAA or Co^{III}-PAM-Lau-PAA as indicated in Fig. 2 and 3. Accumulation of intermediate proteins was not observed during the cleavage of BSA by the DHAB-Lau-PAA derivatives. The small but definite activity of Fe^{III}-DHAB-Lau-PAA or Co^{III}-DHAB-Lau-PAA may be attributed to formation of clusters of the DHAB randomly attached to PAA. It has been reported that an azobenzene derivative or long alkyl chains attached to poly(ethylenimine) form clusters due to hydrophobic interaction.[10] The substantially greater activity of Fe^{III}-PAM-Lau-PAA or Co^{III}-PAM-Lau-PAA compared with Fe^{III}-DHAB-Lau-PAA or Co^{III}-DHAB-Lau-PAA demonstrates that PAM regions constructed by the initial assemblage of **4** by Fe(III) or Co(III) ion prior to the conjugation with PAA are considerably more active than those obtained by the cluster formation.

The two largest intermediate proteins indicated in Fig. 1 were isolated and subjected to *N*-terminal sequencing by Edman degradation as described previously.[1] The sites for cleavage of BSA leading to the formation of the intermediate proteins were the peptide bonds between Arg(24) and Asp(25) and between Trp(237) and Ser(238). The cleavage sites identified previously for the hydrolysis of carboxypeptidase A by Co^{III}-CPBM of **2** were Val(141)-Asp(142) and Met(22)-Asp(23). Among the four cleavage sites identified so far for the PAM-catalyzed protein hydrolysis, three sites involve Asp as the amine portion and one contains Ser as the amine moiety. This suggests selectivity of the PAM derivatives toward Asp and, possibly, Ser.

A molecular mechanics calculation (MM+, HyperChem™) showed that the three-point contact between Asp or Ser peptide with PAM is possible as illustrated in Scheme 2. Mechanistic studies on amide hydrolysis catalyzed by transition metal ions indicated that addition of water to carbonyl group of amide is facilitated by metal ions either through activation of the carbonyl group coordinated to the metal ion or through nucleophilic attack of metal-bound hydroxide ion at the carbonyl group.[2] For amide hydrolysis, protonation of leaving amines is required for facile expulsion of leaving amines. Metal-bound water molecules can act as general acids to protonate the leaving amines.[11] In Scheme 2, the first metal ion stabilizes the tetrahedral intermediate formed by addition of water to the amide and the second metal ion provides metal-bound water that protonates the leaving amine. The extra interaction between Asp carboxylate or Ser hydroxyl with the third metal center of PAM accounts for the selectivity of the PAM derivatives toward Asp or Ser peptides.

Results of the present study demonstrate that proteins are hydrolyzed by the catalytic action of PAMs, excluding the possibility of cleavage of chymotrypsin or carboxypeptidase A due to enhanced self digestion in the presence of the CPBMs of **2**. The PAM-PAAs do not cleave chymotrypsin or carboxypeptidase A whereas the CPBMs are not catalytic in the cleavage of BSA.[12] Activity of the PAMs in protein hydrolysis is far greater than other artificial metallopeptidases reported to date.[13] Highly effective artificial metalloproteases for a target

protein can be, therefore, obtained by proper design of PAM derivatives.

Scheme 2

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5. PAM was originally coined to designate the coordination polymer consisting of monomeric linkages of metal-nitrogen=nitrogen bonds. In view of uncertainties of the structure of the self-assembly of DHAB derivatives obtained in the presence of transition metal ions, the definition of PAM may be extended to include polymeric clusters of metal ions bound to azo ligands.
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