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Fast Hydrolytic Cleavage of Proteins by Coordinatively Polymerized Bilayer Membranes

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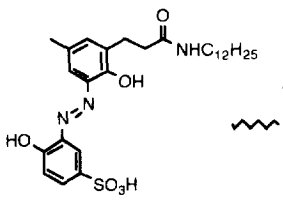
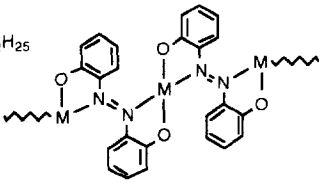
Abstract: Coordinatively polymerized bilayer membranes (CPBMs) obtained by sonication of **1**, an amphiphile containing *o,o'*-dihydroxyazobenzene moiety, in the presence of Co(III) or Fe(III) ion were found to cleave hydrolytically chymotrypsin (ChT) and carboxypeptidase A (CPA). Inactivation of ChT was complete within a few minutes at 4°C and pH 5.5-9.5 when the amphiphile concentration was 5.12 mM. Copyright © 1996 Elsevier Science Ltd

There is much interest in the design of artificial peptidases in view of its importance in molecular recognition and function as well as its relation to biological systems.[1] Metal ions are far more versatile than organic functional groups in catalyzing organic reactions and have been utilized as catalytic groups in many biomimetic catalysts.[2] Participation of multiple metal centers may lead to highly effective catalysis as observed with some metalloenzymes.[3]

o,o'-Dihydroxyazobenzene forms coordination polymers such as **2**.[4] In a previous study, sonication of **1** in the presence of transition metal ions produced CPBMs as schematically illustrated by **3**.[5] In view of the possibility of complexation of the bilayer membranes with proteins and catalytic participation of the metal centers of the coordination polymers in the hydrolysis of peptides, the effects of CPBMs were examined with chymotrypsin$_\alpha$ (ChT) and carboxypeptidase A (CPA) in the present study.



1



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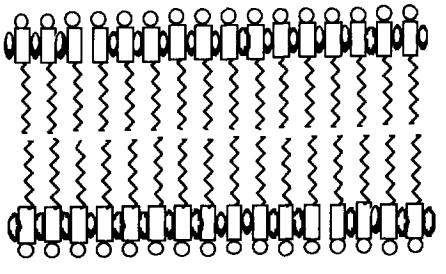

3

Sonication of **1** in the presence of an equivalent of $FeCl_3$ or $[Co(NH_3)_5Cl]Cl_2$ produced a solution of Fe(III)-containing CPBM (Fe-CPBM) or Co(III)-containing CPBM (Co-CPBM) which contained a minor amount of unassembled amphiphile.[5] Similar results of protein cleavage were obtained, however, even when the unassembled amphiphile was removed by gel filtration. When the Co-CPBM of **1** ($[1]_0$ = 5.12 mM) was incubated with ChT (2.12×10^{-5} M) at pH 7.5 and 4°C, ChT was inactivated within a few minutes (Fig. 1). Although ChT was not cleaved by Fe-CPBM of **1**, CPA was inactivated by both Fe-CPBM and Co-CPBM of **1**. The inactivation of CPA was considerably slower compared with ChT (Fig. 1). The activity of ChT was measured by assay with *N*-benzoyl-L-tyrosine-*p*-nitroanilide and that of CPA with *O*-[*trans*-α-(benzoyl-

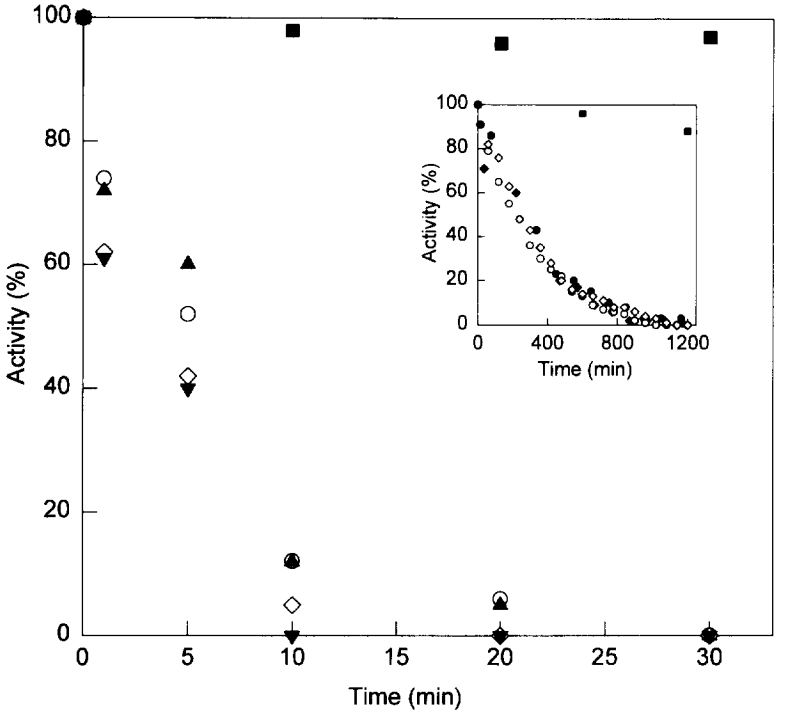

Fig. 1. Spontaneous inactivation of ChT (2.12 X 10^{-5} M) at pH 7.5 and 25°C (■) or inactiviation of ChT measured with Co-CPBM of **1** ($[\mathbf{1}]_o$ = 5.12 mM) at 4°C (▼), 10°C (◇), 15°C (▲), and 25°C (○). Inset, spontaneous inactivation of CPA (1.0 X 10^{-4} M) at pH 7.5 and 25°C (■) and inactivation of CPA measured with Co-CPBM of **1** ($[\mathbf{1}]_o$ =15 mM) at 4°C (○) and 25°C (●) or with Fe-CPBM of **1** ($[\mathbf{1}]_o$ = 15 mM) at 4°C (◇) and 25°C (◆). When the enzymes were incubated with $FeCl_3$ or $[Co(NH_3)_5Cl]Cl_2$ (15 mM) or with the bilayer membrane of **1** ($[\mathbf{1}]_o$ = 15 mM) prepared in the absence of transition metal ions, the reuslts were similar to those of spontaneous inactivation.

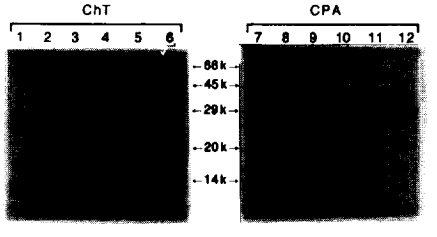

Fig. 2 Separation of cleavage products by electrophoresis after incubation at pH 7.5 and 4°C of ChT (1.0 X 10^{-4} M) for 20 min and of CPA (1.0 X 10^{-4} M) for 1 day. Lanes 6 and 7, standard markers; lanes 1 and 12, incubation with bilayer membrane of **1** ($[\mathbf{1}]_o$ = 15 mM) obtained in the absence of transition metal ions; lanes 5 and 11, incubation with $[Co(NH_3)_5Cl]Cl_2$ (15 mM); lanes 2 and 8, incubation with $FeCl_3$ (15 mM); lanes 4 and 10, incubation with Co-CPBM ($[\mathbf{1}]_o$ = 15 mM); lanes 3 and 9, incubation with Fe-CPBM($[\mathbf{1}]_o$ = 15 mM).

amino)cinnamoyl]-L-β-phenyllactate.[7] The concentration of the CPBM is expressed here in terms of the initially added concentration of amphiphile **1** ($[\mathbf{1}]_o$). Due to the large number of **1** included in the CPBM, the concentration of the CPBM is much lower than the added concentration of ChT or CPA.

When the temperature was raised from 4°C to 25°C, inactivation of ChT became slightly slower whereas that of CPA was not affected appreciably (Fig. 1). When examined at pH 5.5-9.5 (0.05 M buffer), the rate of enzyme inactivation was not affected significantly by pH (not shown).

Loss of enzyme activity can be caused by several reasons in addition to cleavage of polypeptide skeletons. That ChT is indeed inactivated by protein cleavage was evidenced by SDS-PAGE with silver staining.[7] Electrophoresis of the inactivated ChT revealed that the protein was cleaved into fragments smaller than 3 kDa (Fig. 2) within a few minutes at 4°C. ChT is composed of three peptide chains connected by disulfide bonds. The presence of several fragments in lanes 1-3 and 5 of Fig. 2 is due to the cleavage of disulfide bonds of ChT, instead of the cleavage of peptide bonds, under the conditions of electrophoresis in which 10 %(v/v) β-mercpatoethanol was used.

Electrophoresis performed after CPA was fully inactivated indicated several protein fragments (Fig. 2). A major portion of the protein retained the molecular weight similar to the uncleaved CPA. When small peptides are cleaved from the *N*- or *C*-terminal of CPA, the remaining protein would lose the activity although the reduction in molecular weight is small. The fragments of 14, 17 and 20 kDa obtained from inactivation of CPA with Co-CPBM were isolated and subjected to *N*-terminal sequencing by Edman degradation. The cleaved fragments were electroblotted on PVDF membranes for analyses[8] and *N*-terminal sequencing was performed with a Milligen 6600B. The 14 kDa fragment contained the *N*-terminal of CPA_{β}.[9] Sequencing of the 17 and 20 kDa fragments indicated that they were formed by cleavage of Val(141)-Asp(142) and Met(22)-Asp(23), respectively. These two peptide bonds are located on or near the surface of CPA molecule according to the three dimensional structure determined by X-ray crystallography.[10]

When the experiments were performed under aerobic or anaerobic conditions, similar results were obtained for inactivation and cleavage of the proteins. Anaerobic reaction mixtures were prepared under nitrogen, purified by passing it through BTS-catalyst (Fluka) to remove oxygen, with strict exclusion of air by using Schlenk techniques.[11] In addition, protection from light or illumination under day light or a UV-lamp did not affect the results of the present study. After inactivation and degradation into small fragments of ChT (1.02 mM) by Co-CPBM ($[\mathbf{1}]_o$ = 5.12 mM) were complete at pH 6 and 4°C, both Co(III) ion and **1** were fully recovered as judged by the visible spectrum[5] of Co-CPBM and quantitation of **1** by HPLC. In view of the multiple cleavage of ChT, the number of peptide bonds cleaved for each molecule of amphiphile **1** is comparable to or larger than 1. The action of Co-CPBM is, therefore, catalytic. Possibility of self-digestion by ChT or CPA may be excluded on the basis of the control experiments (Figs. 1 and 2). In addition, binding of ChT or CPA on CPBM would compartmentalize the enzyme molecules and reduce the possiblity of contact between two enzyme molecules which is required for self-digestion.

Although catalysis of amide hydrolysis by metal ions has been extensively investigated, most of the catalytic systems involve metal ions tethered to amide bonds.[2] There are only two previous examples of hydrolytic cleavage of proteins by synthetic metal complexes not tethered to the proteins.[3a,b] In these catalytic systems which employed Pd(II) complexes or a macrocyclic Cu(II) complex, however, only partial protein cleavage was achieved even after incubation for several days at 40°C and pH 1.7 for the Pd(II) catalyst or at 50°C and pH 8 for the Cu(II) catalyst.

That the protein cleavage is not affected by exclusion of air and that cleaved protein fragments are successfully degraded by the Edman method have been taken as evidence for the hydrolytic cleavage in the previous study with the macrocyclic Cu(II) complex.[3b] In the present study, the same results were obtained. Furthermore, the action of the CPBMs was found to be catalytic and the protein cleavage was not affected by protection from light. In addition, mechanistic studies have been intensively carried out for the hydrolytic cleavage of amides catalyzed by transition metal ions, especially Co(III) ion.[2b,c] Thus, it is very likely that inactivation of ChT and CPA by the CPBMs involves hydrolytic cleavage of peptide bonds.

The proteolytic activity of the CPBMs reported here is far greater than that of any other artificial

catalysts ever designed such as the Pd(II) or Cu(II) catalysts described above.[1a,b] Recently, a catalytic antibody with peptidic activity has been reported.[1c] The half-life of an amide fully complexed to the catalytic antibody was 49 days at pH 9 and 37°C.

The remarkable proteolytic activity of the CPBMs of **1** implicates that several catalytic elements are operative. ChT and CPA can be complexed to the CPBM. Some peptide linkages may be located in close proximity to the metal centers in the complex formed between the CPBM and the protein. Although the sites for the cleavage of ChT and CPA in the presence of the Fe- or Co-CPBM of **1** are not fully identified, the two cleavage sites revealed for CPA suggest the possibility of cooperation between two adjacent metal centers of the CPBM. If the carboxylate of Asp is bound by a metal center of the CPBM, a molecular mechanics calculation suggested that the scissile peptide linkage may be effectively attacked by hydroxide ion bound to the adjacent metal center.

For both ChT and CPA, the inactivation was not accelerated when the temperature was raised. The mobility of the CPBM complexed with ChT or CPA would be reduced at lower temperatures. This could result in the increased effective concentration of the catalytic center of CPBM relative to the scissile amide linkage of the bound protein. Trypsin, pepsin, carbonic anhydrase, and cytochrome c were cleaved by neither Fe- nor Co-CPBMs of **1**. The marked difference in the activity the CPBMs toward various proteins examined in this study suggests that the proteolytic activity depends on the three-dimensional structure of proteins.

Artificial peptidases have a potentially vast range of practical applications. CPBM is a novel type of artificial metallopeptidase. The structure and reactivity of CPBMs can be tuned by changing amphiphiles and metal ions. Artificial metallopeptidases for a variety of proteins, therefore, might be designed by using CPBMs.

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