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Protein-Cleaving Catalyst Selective for Protein Substrate

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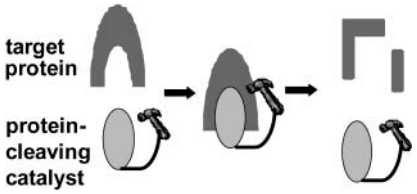
ABSTRACT



A protein-cleaving catalyst specific for a disease-related protein can be used as a catalytic drug. As the first protein-cleaving catalyst selective for a protein substrate, a catalyst for myoglobin was designed by attaching Cu(II) or Co(III) complex of cyclen to a binding site searched by a combinatorial method using peptide nucleic acid monomers as building units.

In search of new drugs, efforts are made to design molecules that specifically block the activity of disease-related proteins such as enzymes, receptors, and ion channels.[1−4] Even if a drug molecule is bound to its target protein very strongly, at least an equivalent amount of the drug is needed to inactivate the protein. If a disease-related protein is deactivated upon cleavage of its polypeptide backbone by a synthetic catalyst, the catalyst can be used as a drug. Because a catalytic amount of the drug can destroy the protein, the dosage can be reduced substantially by using the catalyst. Designing protein-cleaving catalysts specific for target proteins, therefore, would become a powerful tool in drug discovery.

Several synthetic catalysts with proteolytic activity lacking substrate selectivity have been reported.[5−17] Both organic functional groups and metal complexes have been exploited as the catalytic groups in those artificial proteases. A protein-cleaving catalyst with high substrate selectivity and catalytic rate would be obtained by connecting a binding site that recognizes the target protein to a catalytic group. On complexation of the protein-cleaving catalyst to the target protein, the effective molarity[18] of the catalytic group toward a peptide linkage of the target protein can increase to a sufficiently high level to allow facile cleavage of the peptide bond. To suppress cleavage of nontarget proteins, the

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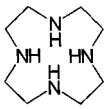
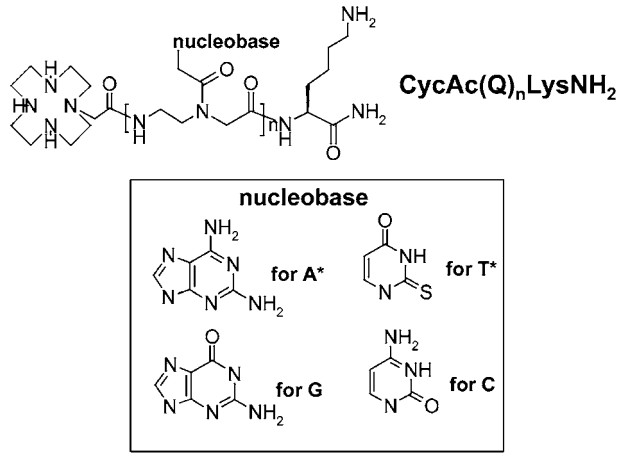
catalytic center should have very low protein-cleaving activity when unattached to the binding site. In the present study directed toward the first protein-cleaving catalyst selective for a target protein, we chose metal complexes of cyclen (Cyc) as the catalytic center since a previous study[11] indicated that the Cu(II) complex of Cyc has very low proteolytic activity unless activated by additional catalytic elements.



As the binding site of the specific protein-cleaving catalyst, small organic compounds such as inhibitors or antagonists already reported to have high affinity toward the target protein may be used. Alternatively, a new binding site may be searched by the combinatorial approach. The catalyst obtained by the latter method does not necessarily bind to the active site of the protein.

In the present study, we constructed a combinatorial library of Cyc derivatives containing analogues of peptide nucleic acid (PNA)[19,20] (Figure 1) to search the binding site of a protein-cleaving catalyst. PNA was employed as the potential binding site in view of protein recognition[21] by derivatives of purine. The library can be presented as CycAc(Q)$_n$LysNH$_2$ where Q is PNA monomer A*, G, T*, or C. PNA contains nucleobases that can be used for base-pairing with nucleobases of DNA. We used modified nucleobases A* and T* instead of A and T. A* and T* recognize T and A, respectively. A* and T*, however, do not recognize each other.[22] Base-pairing among PNA mixtures present in the library, therefore, can be suppressed by using A* and T*.



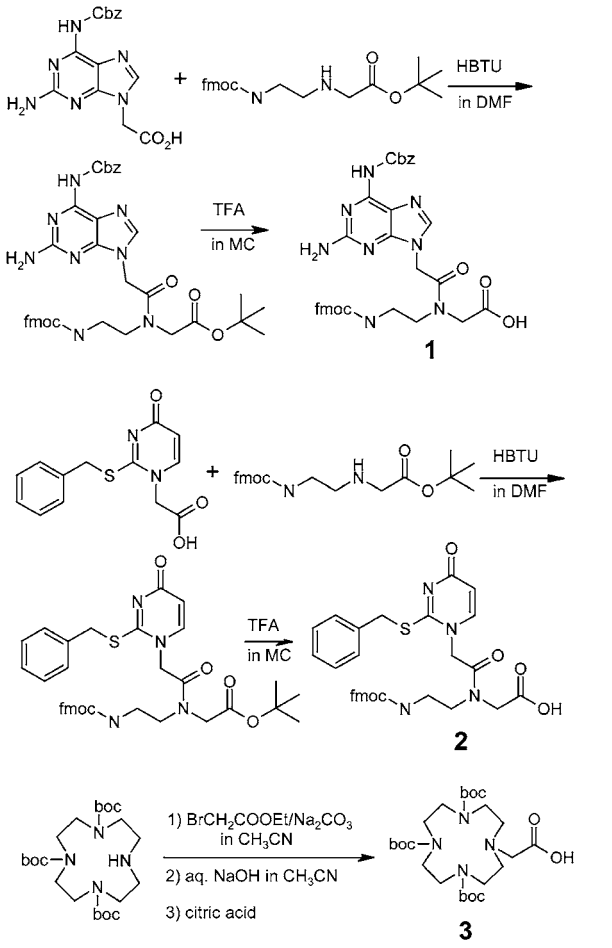
CycAc—A*-A*-T*-T*-C-G-A*-A*-C-LysNH$_2$ (**4**)

CycAc—C-A*-T*-T*-C-G-A*-A*-C-LysNH$_2$ (**4a**)

Figure 1. Structures of the combinatorial library of Cyc derivatives containing PNA oligomers, ligand (**4**) of the catalyst selected from the library, and an analogue (**4a**) of **4**.

The PNA derivatives were synthesized by automated synthetic procedures using an Expedite model 8909 Nucleic Acid Synthesis System with the fmoc-derivatives of A*, T*, G, C, and L-Lys as well as (boc)$_3$CycAcOH. PNA monomers G and C as well as L-lysine protected with the fmoc group were purchased from commercial sources whereas A* (**1**) and G* (**2**) protected with the fmoc group as well as (boc)$_3$CycAcOH (**3**) were synthesized according to the synthetic pathways summarized in Scheme 1. In the construction of the library, it was assumed that the carboxyl groups of the fmoc derivatives of A*, T*, G, and C have identical reactivity in coupling with the amino ends of PNA oligomers attached to the polymer support. Purity of various PNA derivatives was checked by MALDI-TOF MS analysis by using a Voyager-DE STR biospectrometry workstation model.

Scheme 1



The library of CycAc(Q)$_n$LysNH$_2$ (total concentration: ca. 70 μM) was mixed with an aqueous solution of Cu(II)Cl$_2$ (350 μM) to generate the library of Cu(II)CycAc(Q)$_n$LysNH$_2$ where Cu(II) is bound to the Cyc moiety. Cu(II) forms a strong complex with Cyc (log K_f = 16.8 at pH 7).[23] With the Cu(II)Cyc library containing 7- or 8-mer PNAs, no evidence was obtained for cleavage of proteins (10 μM) such as bovine serum albumin, γ-globulin, elongation factor P,

gelatin A, gelatin B, and horse heart myoglobin (Mb) at 37 °C and pH 7 as checked by electrophoresis (SDS−PAGE). Methods for kinetic measurement of protein cleavage by SDS−PAGE are described elsewhere.[5−7,11] The Cu(II)Cyc library containing 9-mer PNAs clearly showed activity for cleavage of Mb. Four groups of library with the known PNA monomers positioned next to Cu(II)Cyc were subsequently synthesized and tested for their activity and A* was identified as the best monomer for that position. By repeating the search for the rest of the nine positions occupied by PNA monomers, **4** (Figure 1) was chosen as the ligand of the best catalyst. The binding site for Mb was, therefore, obtained by using the pyrimidine and purine bases of **4**.

The stock solution of Cu(II) complex of **4** was prepared by adding an aqueous solution of $CuCl_2$ to **4** (1.2 equiv) at pH 6.0. The degradation of Mb by Cu(II)**4** was followed by electrophoresis (SDS−PAGE). An example of the plot of [Mb] (total concentration of uncleaved Mb; estimated from the electrophoretic bands of Mb) against time is illustrated in Figure 2. The time-dependent decrease in [Mb] was fitted

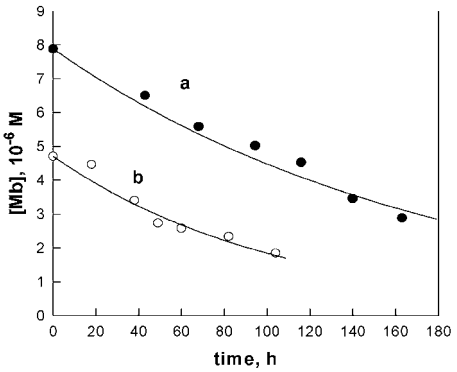


Figure 2. Decrease in [Mb] during incubation of Mb with Cu(II)**4** (●; curve a, $[Mb]_o = 7.9\ \mu M$, $[Cu(II)\mathbf{4}]_o = 2.0\ \mu M$) or Co(III)**4** (○; curve b, $[Mb]_o = 4.7\ \mu M$, $[Co(III)\mathbf{4}]_o = 0.47\ \mu M$) at pH 7.5 and 37 °C. The curves were obtained as indicated in the text: $k_o = 5.7 \times 10^{-3}\ h^{-1}$ for curve a and $9.4 \times 10^{-3}\ h^{-1}$ for curve b.

to pseudo-first-order kinetic equations to obtain pseudo-first-order rate constant (k_o).

To check whether other metal ions complexed to **4** cleave Mb, metal ions such as Co(III), Fe(III), Hf(IV), Pt(IV), Zr(IV), Pd(II), and Ce(IV) were added to **4** to generate the respective complexes. Due to the kinetic inertness[24] of Co(III) complexes, direct insertion of Co(III) ion to the chelating ligands is not easy. Instead, the Co(III) complex of **4** was obtained by incorporating Co(II) ion to **4** and then oxidizing the complexed Co(II) ion in methanol according to the literature[25] procedure: for Co(III)**4**, MS (MALDI-TOF) *m*/*z* 2908.44 $(M + H)^+$ ($C_{111}H_{153}N_{64}O_{25}S_2Co$ calcd 2908.51). Among the metal ions tested, Co(III) manifested the protein-cleaving activity upon complexation to **4**. It is noteworthy that Co(III) complexes may be more suitable for medical uses compared with Cu(II) complexes since metal transfer to metal-abstracting materials in living body should be substantially slower for the Co(III) complexes.

An example of degradation of Mb by Co(III)**4** is illustrated in Figure 2. Although the structure of **4** was obtained by using the Cu(II) complex, detailed kinetic analysis was performed with the Co(III) complex due to the higher catalytic activity of the Co(III) complex. The dependence of k_o on C_o (the initially added concentration of the catalyst) measured at pH 7.5 is illustrated in Figure 3. Although the

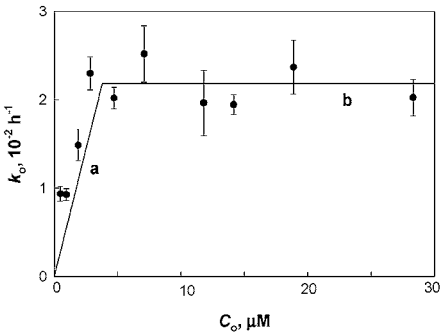


Figure 3. Plot of k_o against C_o for cleavage of Mb ($[Mb]_o = 4.7\ \mu M$) by Co(III)**4** at pH 7.5 and 37 °C (0.05 M buffer; addition of 0.5 M NaCl did not affect the rate data appreciably). Straight lines a ($C_o < S_o$) and b ($C_o \geq S_o$) stand for v_o/S_o (v_o: initial velocity) and k_o, resepctively, predicted by Michaelis−Menten scheme under the condition of $C_o \gg K_m$.

plot of ln [Mb] against time was fitted to a straight line to obtain k_o, Michaelis−Menten scheme predicts that the kinetic behavior does not conform to first-order kinetics when C_o is smaller than $[Mb]_o$ (the initially added concentration of Mb) even when $C_o \gg K_m$. Moreover, rate data based on electrophoretic measurement are not very accurate. These may be related to the scattered data points of Figure 3 at $C_o < [Mb]_o$.

Although the kinetic data are somewhat scattered, the two straight lines drawn in Figure 3 intersect at $C_o = [Mb]_o$. This intersection agrees with strong binding of Mb to Co(III)**4**: in terms of Michaelis−Menten parameters, $K_m \ll C_o$ and, thus, $K_m \ll 5\ \mu M$. Furthermore, k_o measured with C_o greater than $[Mb]_o$ corresponds to k_{cat}.

The k_{cat} values thus measured at various pHs are illustrated in Figure 4. If ionization of Mb or **4** is disregarded, the pK_a values (5.50, 8.68) estimated from analysis of the bell-shaped pH profile may be assigned to the ionization of water

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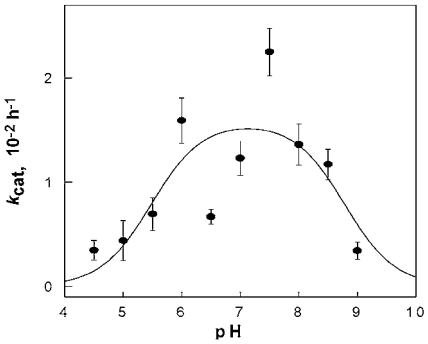

Figure 4. pH dependence of k_{cat} for cleavage of Mb by Co(III)**4** at 37 °C. The bell-shaped curve was obtained by analyzing the data by treating the Co(III)**4**−Mb complex as a diprotonic acid ($pK_{a1} = 5.50$, $pK_{a2} = 8.68$) and by assuming that the monoprotonated species is reactive.

molecules coordinated to Co(III) ion of Co(III)**4**−Mb complex. It is noteworthy that the catalyst is most active at the physiological pH. The k_{cat} measured at pH 7.5 and 37 °C corresponds to the half-life of 30 h. This may be compared with the half-life[26] of 200 y for spontaneous hydrolysis of unactivated amides measured under identical conditions. On optimization of the linker connecting Co(III)-Cyc and the PNA moiety of **4**, k_{cat} would be raised further.

The MALDI-TOF mass spectrum (Figure 5) of a reaction

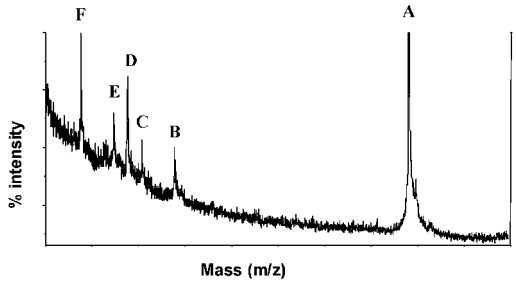

Figure 5. MALDI-TOF mass spectrum taken after incubation of Mb (12 μM) with Co(III)**4** (3.5 μM) at pH 6.0 and 37 °C for 85 h. Mass values are 16953 (A), 9892 (B), 8909 (C), 16953/2 (D), 8045 (E), and 7074 (F); A and D are peaks for Mb (MW 16953), and two pairs (B/F and C/E) are for proteins formed by cleavage of Mb.

mixture obtained by incubation of Mb with Co(III)**4** disclosed that Mb was dissected into two pairs of proteins. Possible sites of the protein cleavage by Co(III)**4** are: Leu89-Ala90 (producing fragments with MW 7077 and 9894) and Leu72-Gly73 (producing fragments with MW 8057 and 8914). Attempts to identify the N-terminal sequences of the cleavage products were unsuccessful since individual protein fragments were not separated cleanly by electrophoresis. It is not clear at present whether the two cleavage sites involve different Co(III)**4**−Mb complexes or originate from an identical one. More information on binding mode of the PNA portion of the catalysts would be obtained by crystallographic studies.

When other proteins such as albumin, γ-globulin, elongation factor P, gelatin A, and gelatin B were incubated with Cu(II)**4** or Co(III)**4**, protein cleavage was not observed. When Mb was treated with $CuCl_2$, Cu(II)Cyc, or Co(III)Cyc instead of Cu(II)**4** or Co(III)**4**, Mb was not degraded. An analogue of Co(III)**4** was prepared where the PNA residue next to the CycAc unit is C instead of A* as indicated by **4a**. No catalytic activity was observed for Co(III)**4a** and Cu(II)**4a** in the cleavage of Mb. The degree of intramolecular cleavage of amide bonds of Cu(II)**4** or Co(III)**4** was not significant when followed for several days by measuring the MALDI-TOF mass spectrum and the catalytic activity, probably due to the steric strain involved in the attack of the metal center at an internal amide bond.

Up to 2.5 or 6 molecules of Mb were cleaved by each molecule of Cu(II)**4** or Co(III)**4**, respectively, for the data of Figure 2, indicating the catalytic nature of the action of Cu(II)**4** and Co(III)**4**. The number of Mb molecules cleaved per catalyst molecule was small due to the small value of k_{cat} as a consequence of the low effective molarity of the catalytic center in the Mb-catalyst complex. Catalytic turnover by Co(III)**4**, despite the exchange-inertness of Co(III) ion, indicates that the protein-cleavage products dissociate from the Co(III) ion effectively. In this regard, carboxylates bound to Co(III) complexes can be freed hydrolytically through C−O bond cleavage.[27]

The rate for cleavage of Mb by Cu(II)**4** or Co(III)**4** was unaffected by the removal of O_2 from the reaction mixtures. These results and previous observations[8,13,15] for hydrolytic cleavage of peptide bonds by Cu(II) complexes of tetraaza ligands and Co(III) complexes suggest the hydrolytic nature of cleavage of Mb by Cu(II)**4** and Co(III)**4**.

The present study demonstrates that protein-cleaving catalysts specific for target proteins can be designed by choosing appropriate binding sites, linkers, and catalytic units. The activity of a protein-cleaving catalyst would be improved by the increase in its affinity to the target protein and by proper orientation of the catalytic group in the catalyst-protein complex. By following the general principles discovered here, it would be possible to design protein-cleaving catalysts specific for selected proteins. If the selected targets are disease-related proteins, the protein-cleaving catalysts can serve as catalytic drugs.

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Supporting Information Available: Experimental procedures for synthesis of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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