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Toward Protein-Cleaving Catalytic Drugs: Artificial Protease Selective for Myoglobin

Joong Won Jeon, Sang Jun Son, Chang Eun Yoo, In Seok Hong and Junghun Suh*

School of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-747, South Korea

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Abstract—A protein-cleaving catalyst highly selective 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 (Mb) was designed by attaching the 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. Various linkers were inserted between the catalytic Co(III) center and the binding site of the Mb-cleaving catalyst. Kinetic data revealed catalytic turnover of the Mb cleavage by the Cu(II) or Co(III) complex. MALDI-TOF MS revealed cleavage of the polypeptide backbone of Mb at selected positions. N-Terminal sequencing of the cleavage products identified the cleavage site and provided evidence for the hydrolytic nature of the Mb cleavage. Various chelating ligands were tested as the ligand for the Co(III) center of the Mb-cleaving catalyst. Among the nine chelating ligands examined, only cyclen and its triaza-monooxo analogue manifested catalytic activity.

Introduction

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 highly selective for target proteins, therefore, would become a powerful tool in drug discovery. To establish the idea of protein-cleaving catalytic drugs, it is essential to synthesize the first artificial protease selective for a target protein.

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 non-target proteins, the catalytic center should have very low protein-cleaving activity when unattached to the binding site.

As the catalytic moiety of a target-selective artificial protease, either a purely organic catalytic center or a metal complex may be used. Each of the organic artificial proteases reported to date uses an artificial active site comprising two or more organic catalytic groups. On the other hand, a single metal complex can often act as an effective catalytic site for artificial metalloproteases. In the present study, we chose to use metal complexes as the catalytic center of the first target-selective artificial protease.

As the binding site of the target-selective artificial protease, 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

*Corresponding author. Tel.: +82-2-886-2184; fax: +82-2-874-3704; e-mail: jhsuh@snu.ac.kr

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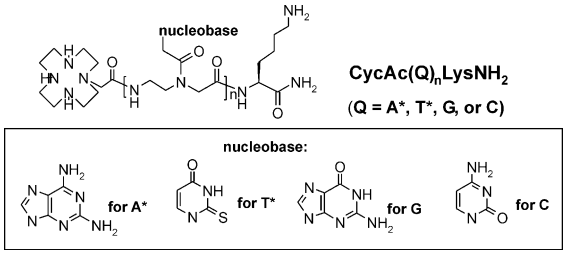
does not necessarily bind to the active site of the protein. In the present study, we adopted the combinatorial method to obtain the binding site of the target-selective artificial protease using a metal complex as the catalytic site. In this article, selection of the lead catalyst selective for myoglobin (Mb) and effects of variation in the shape of the linker connecting the binding site and the catalytic site as well as the structure of the chelating ligand of the catalytic site are described.[19]

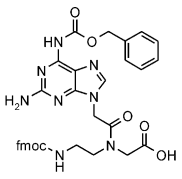
Results

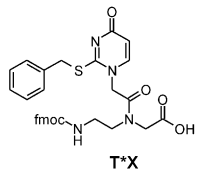
In the present study directed toward the first protein-cleaving catalyst selective for a target protein, we chose metal complexes of cyclen (Cyc) as the catalytic center since a previous study[11] indicated that the Cu(II) complex of Cyc has very low proteolytic activity unless activated by additional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

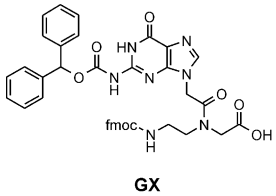
In the present study, we constructed a combinatorial library of Cyc derivatives containing analogues of peptide nucleic acid (PNA)[21,22] to search the binding site of a protein-cleaving catalyst. Since organic functional groups of some proteins are exploited in recognition of nucleobases of nucleic acids,[23] nucleobases of PNAs may be conversely used in recognition of proteins. 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.[24] Base-pairing among PNA mixtures present in the library, therefore, can be suppressed by using A* and T*.

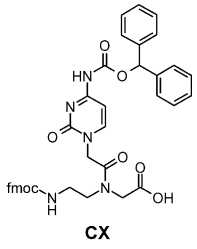


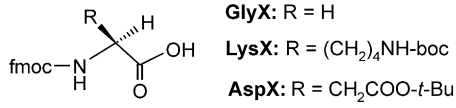
The PNA derivatives were synthesized by automated synthetic procedures with the fmoc-derivatives of A*, T*, G, and C (**A*X**, **T*X**, **GX**, and **CX**, respectively) as well as N^{α}-fmoc-N^{ε}-boc-L-Lys (**LysX**) and the boc-derivative of CycAcOH (**1X**). The library of Cyc-containing PNA oligomers with the general structure of CycAc(Q)$_n$LysNH$_2$ was constructed by the Split-Pool method. In the construction of the library, it was assumed that the carboxyl groups of **A*X**, **T*X**, **GX**, and **CX** have identical reactivity in coupling with the amino groups linked to the polymer support.

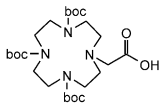












The library of CycAc(Q)$_n$LysNH$_2$ (total concentration: ca. 70 μM) was mixed with an aqueous solution of Cu(II)Cl$_2$ (350 μM) to generate the library of Cu(II)CycAc(Q)$_n$LysNH$_2$ where Cu(II) is bound to the Cyc moiety. Cu(II) forms a strong complex with Cyc (log K_f = 16.8 at pH 7).[25] With the 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 Mb at 37 °C and pH 7 as checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The Cu(II)Cyc library containing 9-mer PNAs, however, 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, **1** (Fig. 1) was chosen as the ligand of the best catalyst. The binding site for Mb was, therefore, obtained by using the functional groups in **1** such as the pyrimidine and purine bases and amide groups. The structures of **1** and its analogues (**2**–**17**) synthesized in the present study are illustrated in Figure 1.

The position of the metal complex of Cyc relative to the PNA 9-mer was varied. Cyc was attached either to the *C*- (**2**) or the N-terminus of the PNA oligomer. When Cyc was attached to the N-terminus, the catalytic site contained either one (**3**–**8**) or two (**9**) Cyc units. To suppress the conformational freedom of the Cyc-acetyl moiety of **1**, methyl group was attached to the acetyl

portion of the Cyc-acetyl moiety leading to **3**. Amino acids such as Gly, L-Asp, L-Pro, L-Lys, and β-Ala were inserted between the Cyc-acetyl moiety and the PNA portion of **1** to obtain **4**–**8**. For synthesis of **2**–**9** by the automated procedures, **2Y**, **3X**, and **9X** synthesized in this study as summarized in Schemes 1–3 and **GlyX**, **AspX**, **ProX**, and **BalX** (*N*-fmoc derivatives of Gly, L-Asp β-*t*-butyl ester, L-Pro, and β-Ala, respectively) purchased from commercial sources were used as additional building blocks.

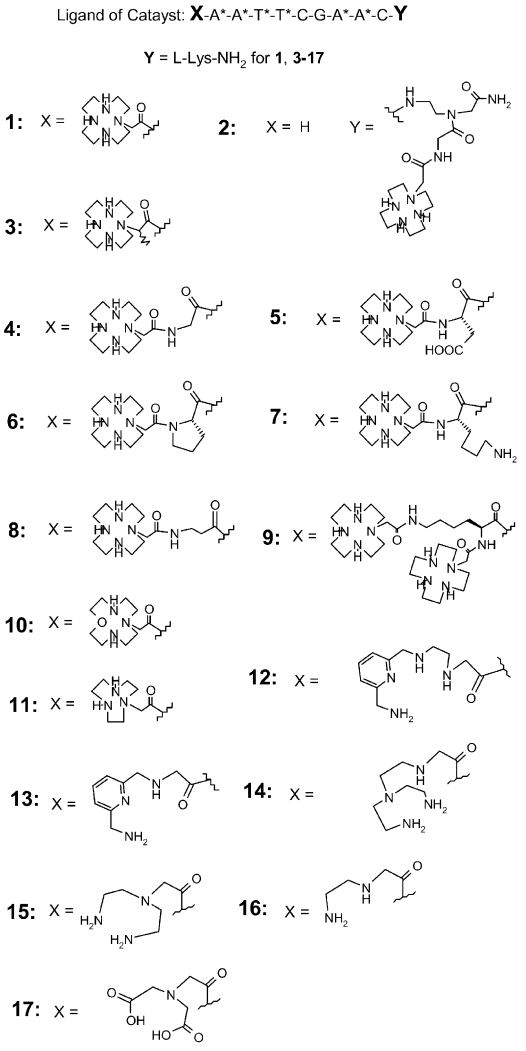
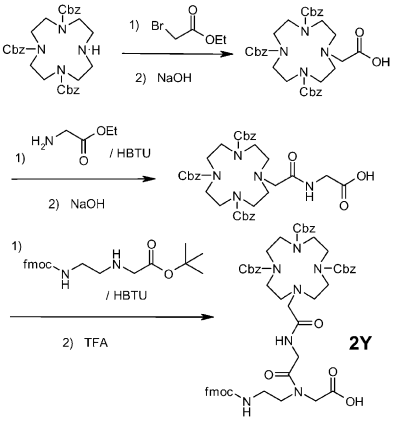


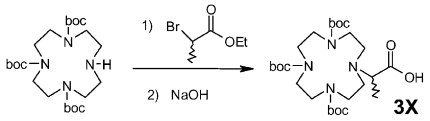
Figure 1. Structures of **1**–**17**.



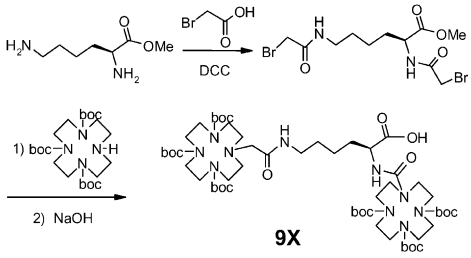
Scheme 1. Synthesis of **2Y**.

In view of the wide range of potential application of the target-specific protein-cleaving catalysts to drug design, whether the Cyc moiety of **1** can be replaced by other chelating ligands is important. In the present study, therefore, **10**–**17** were tested to evaluate effectiveness of various alternative chelating ligands. For synthesis of **10**–**17** by the automated procedures, **10X**–**17X** were synthesized as summarized in Schemes 4–11 and used as additional building blocks.

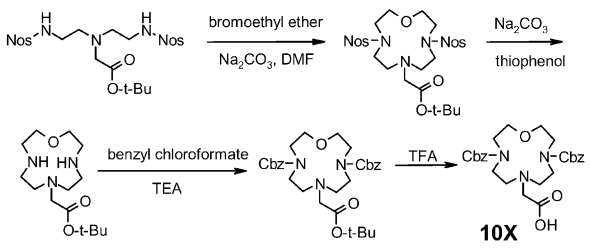
The stock solution of Cu(II) complex of **1** was prepared by adding an aqueous solution of $CuCl_2$ to **1** (1.2 equiv) at pH 6.0. Due to the kinetic inertness[26] of Co(III) complexes, direct insertion of Co(III) ion to the chelating ligands is not easy. Instead, the Co(III) complexes of **1**–**14** were generated in situ by incorporating Co(II) ion to the respective Cyc derivatives and then oxidizing the complexed Co(II) ion in methanol according to the literature[27] procedure. For **15**–**17**, Co(III) complexes were not obtained by the method employed in this study. Binding of Co(III) ion to aza ligands was confirmed by appearance of λ_{max} around 510 nm in Vis spectrum and the matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF MS).



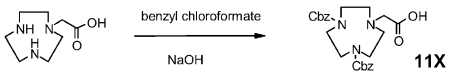
Scheme 2. Synthesis of **3X**.



Scheme 3. Synthesis of **9X**.



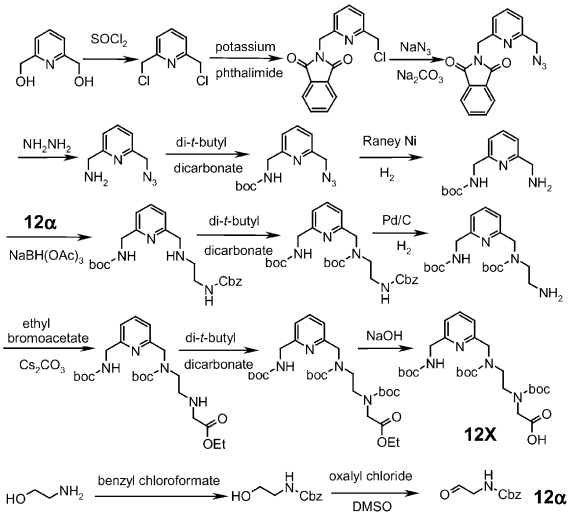
Scheme 4. Synthesis of **10X**.



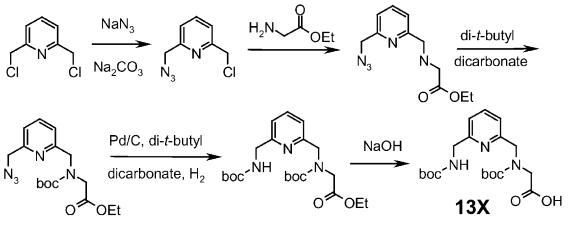
Scheme 5. Synthesis of **11X**.

Reaction rates for cleavage of protein substrates were followed by SDS-PAGE according to the methods described elsewhere.[5–7,11] 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 to pseudo-first-order kinetic equations to obtain pseudo-first-order rate constant (k_o).

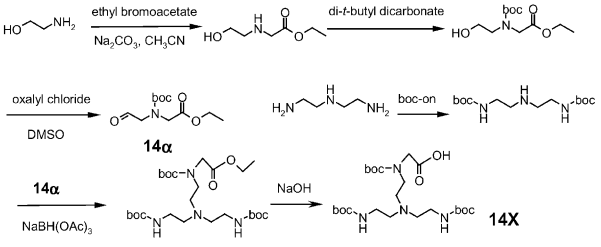
Although the structure of **1** was obtained by using the Cu(II) complex, detailed kinetic analysis was performed with the Co(III) complex due to the higher catalytic activity of the Co(III) complex revealed by the kinetic data of Figure 2. The dependence of k_o on C_o (the initially added concentration of the catalyst) measured with Co(III)**1** at pH 7.5 is illustrated in Figure 3. Although the 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 $C_o >> K_m$. Moreover, rate data based on electrophoretic measurement are not very accurate. These may be related to the scattered data points of Figure 3 at $C_o < [Mb]_o$.



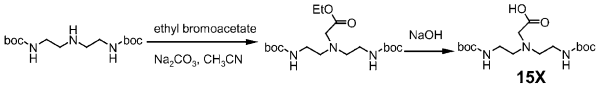
Scheme 6. Synthesis of **12X**.



Scheme 7. Synthesis of **13X**.



Scheme 8. Synthesis of **14X**.

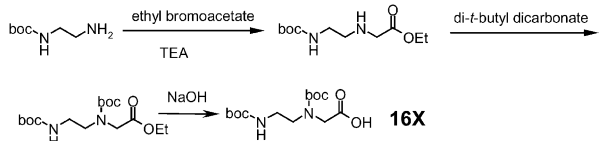


Scheme 9. Synthesis of **15X**.

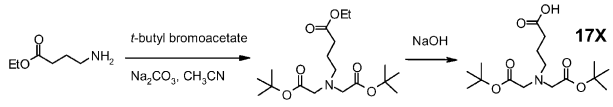
Although the kinetic data are somewhat scattered, the two straight lines drawn in Figure 3 intersect at $C_o = [Mb]_o$. This intersection agrees with strong binding of Mb to Co(III)**1**: in terms of Michaelis–Menten parameters, $K_m << C_o$ and, thus, $K_m << 5\,\mu M$. Furthermore, k_o measured with C_o greater than $[Mb]_o$ corresponds to k_{cat}.

For the Co(III) complexes of **2**–**14**, activity for the Mb cleavage was observed only with **3**, **6**, **7**, **9**, and **10**. The dependence of k_o on C_o for the Co(III) complexes of **3**, **6**, **7**, **9**, and **10** was the same as that observed with that of **1**. For **3**, **6**, **7**, **9**, and **10**, therefore, $K_m << C_o$ and $K_m << 10\,\mu M$ when analyzed in terms of Michaelis–Menten scheme. Furthermore, k_o measured with C_o greater than $[Mb]_o$ corresponds to k_{cat}.

The k_{cat} values thus measured at various pHs for the cleavage of Mb by Co(III)**1** and Co(III)**10** are illustrated in Figures 4 and 5. The pK_a values reflected in the bell-shaped pH profiles can be assigned to the



Scheme 10. Synthesis of **16X**.



Scheme 11. Synthesis of **17X**.

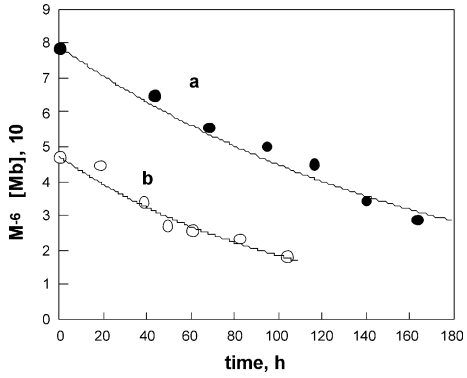


Figure 2. Decrease in [Mb] during incubation of Mb with Cu(II)**1** (●; curve a, $[Mb]_o = 7.9\,\mu M$, $[Cu(II)\mathbf{1}]_o = 2.0\,\mu M$) or Co(III)**1** (○; curve b, $[Mb]_o = 4.7\,\mu M$, $[Co(III)\mathbf{1}]_o = 0.47\,\mu M$) at pH 7.5 and 37 °C. The curves were obtained as indicated in the text: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

ionization of functional groups of the complex formed between Mb and Co(III)**1** or Co(III)**10**. It is noteworthy that the catalyst is most active at the physiological pH. The k_{cat} values measured at the optimum pH for the Co(III) complexes of **1**, **3**, **6**, **7**, **9**, and **10** are summarized in Table 1.

MALDI-TOF MS (Fig. 6) of a reaction mixture obtained by incubation of Mb with Co(III)**1** disclosed that Mb was dissected into two pairs of proteins. Attempts to identify the N-terminal sequences of the cleavage products were unsuccessful since individual protein fragments were not separated cleanly by electrophoresis. For the cleavage of Mb by the Co(III) complexes of **3**, **6**, **7**, **9**, and **10**, however, MALDI-TOF MS taken for the solutions obtained by incubation of Mb with the Co(III) complexes revealed single cleavage site, as exemplified by the spectrum illustrated in Figure 7. The molecular weights of protein fragments disclosed by MALDI-TOF MS are summarized in Table 2 together with the possible cleavage sites for the protein fragments deduced from the primary structure of Mb.

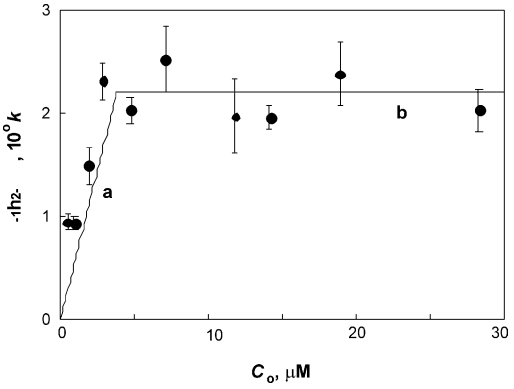


Figure 3. The plot of k_o against C_o for cleavage of Mb ($[Mb]_o = 4.7\,\mu M$) by Co(III)**1** at pH 7.5 and 37 °C (0.05 M buffer; addition of 0.5 M NaCl did not affect the rate data appreciably). Straight lines a ($C_o < S_o$) and b ($C_o \geq S_o$) stand for v_o/S_o (v_o: initial velocity) and k_o, resepctively, as predicted by Michaelis–Menten scheme under the condition of $C_o >> K_m$.

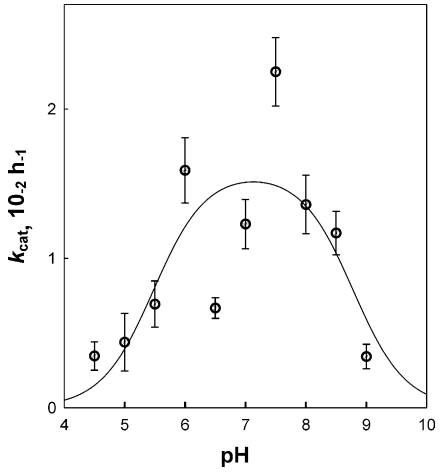


Figure 4. pH dependence of k_{cat} for cleavage of Mb by Co(III)**1** at 37 °C. The bell-shaped curve was obtained by analyzing the data by treating the Co(III)**1**-Mb complex as a diprotonic acid ($pK_{a1} = 5.5$, $pK_{a2} = 8.7$) and by assuming that the monoprotonated species is reactive.

Since only two protein fragments were formed by Co(III)**3**, the product solution obtained by incubation of Mb (9.8 μM) with Co(III)**3** (8.5 μM) for 7 days at pH 8.0 and 37 °C was subject to N-terminal sequencing by Edman degradation. Results of the N-terminal sequencing indicated that Leu_{89}-Ala_{90} was the cleavage site in agreement with the cleavage site proposed on the basis of MALDI-TOF MS results. Moreover, the successful N-terminal sequencing indicates that the amino group was generated after peptide cleavage in support of the hydrolytic nature of the Mb cleavage.

When Fe(III), Hf(IV), Pt(IV), Zr(IV), Pd(II), or Ce(IV) ion was added to **1** and then incubated with Mb, Mb cleavage was not observed. When other proteins such as

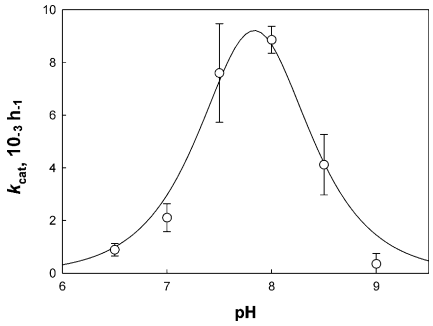


Figure 5. pH dependence of k_{cat} for cleavage of Mb by Co(III)**10** at 37 °C. The bell-shaped curve was obtained by treating the Co(III)**10**–Mb complex as a diprotonic acid ($pK_{a1} = 7.4$, $pK_{a2} = 8.3$) and by assuming that the monoprotonated species is reactive.

Table 1. Values of k_{cat} measured at optimum pH and 37 °C for the Co(III) complexes of **1**, **3**, **6**, **7**, **9**, and **10**

Catalyst	k_{cat} (10^{-3} h^{-1})[a]	Optimum pH
Co(III)**1**	22	7.5
Co(III)**3**[b]	2.8	7.5
Co(III)**6**	2.4	7.5
Co(III)**7**	2.4	7.5
Co(III)**9**	4.4	7.5
Co(III)**10**	8.9	8.0

[a]Relative standard deviation: 10–20%.
[b]For the two enantiomers of **3X**, only one may lead to an active artificial protease. Thus, the observed k_{cat} value may underestimate the actual catalytic capability.

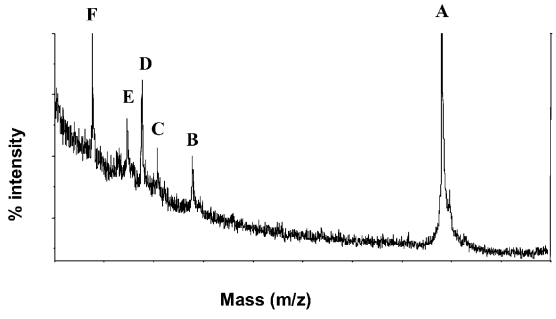


Figure 6. MALDI-TOF MS taken after incubation of Mb (12 μM) with Co(III)**1** (3.5 μM) at pH 6.0 and 37 °C for 85 h. Mass values (m/z) are 16953 (A), 9892 (B), 8909 (C), 16953/2 (D), 8045 (E), and 7074 (F); A and D are peaks for Mb (M.W. 16953) and two pairs (B/F and C/E) are for proteins formed by cleavage of Mb.

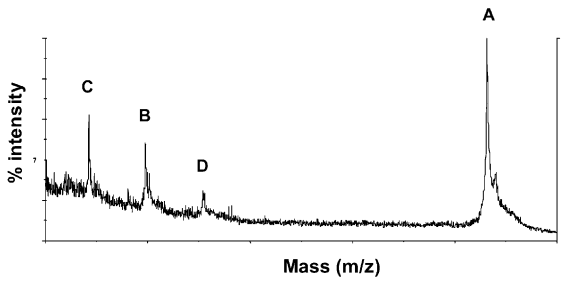


Figure 7. MALDI-TOF MS taken after incubation of Mb (9.8 μM) with Co(III)**7** (9.8 μM) at pH 7.5 and 37 °C for 96 h. Mass values (m/z) are 16953 (A), 16953/2 (B), 7068 (C), and 9888 (D); A and B are peaks for Mb and C and D are for proteins formed by cleavage of Mb.

Table 2. Molecular weights of protein fragments disclosed by MALDI-TOF MS and cleavage sites proposed to account for the protein fragments

Catalyst	m/z of fragments	Cleavage site proposed on the basis of m/z values[a]
Co(III)**1**	7074, 9891, 8045, 8909	Leu_{89}-Ala_{90}, Leu_{72}-Gly_{73}
Co(III)**3**	7066, 9897	Leu_{89}-Ala_{90}
Co(III)**6**	7066, 9898	Leu_{89}-Ala_{90}
Co(III)**7**	7068, 9888	Leu_{89}-Ala_{90}
Co(III)**9**	7068, 9884	Leu_{89}-Ala_{90}
Co(III)**10**	7075, 9896	Leu_{89}-Ala_{90}

[a] M_r of protein fragments to be obtained: 7059 and 9893 for cleavage at Leu_{89}-Ala_{90}, 8056 and 8896 for cleavage at Leu_{72}-Gly_{73}.

albumin, γ-globulin, elongation factor P, gelatin A, and gelatin B were incubated with Cu(II)**1** or Co(III)**1**, protein cleavage was not observed. When Mb was treated with $CuCl_2$, Cu(II)Cyc, or Co(III)Cyc, Mb was not degraded. An analogue of **1** was prepared where the PNA residue next to the CycAc unit is C instead of A* as indicated by **18**. No catalytic activity was observed for Co(III)**18** and Cu(II)**18** in the cleavage of Mb. The degree of cleavage of amide bonds of Cu(II)**1** or Co(III)**1** was not significant when followed for several days under the conditions of kinetic measurements of the present study by measuring MALDI-TOF MS and the catalytic activity for Mb cleavage.[28] The rate for cleavage of Mb by Cu(II)**1** or Co(III)**1** was unaffected by the removal of O_2 from the reaction mixtures.

CycAc–C–A*-T*-T*-C-G–A*-A*-C–$LysNH_2$ **18**

Discussion

Libraries of PNA oligomers with the general structure of CycAc(Q)$_n$$LysNH_2$ were prepared with an automated synthesizer by the Split-Pool method. In each step of attaching a new PNA monomer to the amino group provided by the polymer support, equal amounts of **A*X**, **T*X**, **GX**, and **CX** were added assuming equal reactivity of the four monomers in the coupling reaction. The library of CycAc(Q)$_n$$LysNH_2$ contains a large number of PNA oligomers. The theoretical number of the PNA oligomers contained in the library is 4^n (262,144 when $n=9$), but the actual number might be considerably smaller than this number because the assumption of the equal reactivity may not be valid.

To test the proteolytic activity of the library, the Cyc moieties of the library were converted to the Cu(II) complexes and the mixture of Cu(II)CycAc(Q)$_n$$LysNH_2$ was incubated with various proteins. Although the nucleobases of A* and T* do not recognize each other, those of G and C can form complexes with each other. A significant portion of the members of the library can form duplexes with other members containing complementary sequences, especially when the G/C content of the PNA oligomer is high. Those duplexes might have low affinity for the target protein and, thus, have limited proteolytic activity. Although a large number of PNA oligomers were generated by the combinatorial method, only a small portion may exist as unfolded unimolecular identities in the buffer solution. In **1**, which contains a PNA oligomer chosen as the most active sequence, six nucleobases out of nine are A* or T*.

Compounds **1**–**18** synthesized with the automated synthesizer were contaminated with small amounts of impurities as exemplified by the MALDI-TOF MS of Co(III)**1** illustrated in Figure 8. In most of the Co(III) complexes, the Co(III) ion do not considerably dissociate from the Cyc portion under the conditions of measurement of MALDI-TOF MS. Purification of the PNA derivatives by HPLC did not remove the MALDI-TOF MS peaks of the impurities significantly. Except for **2**, Cyc moiety was attached in the last synthetic step and, therefore, the contaminants do not have the Cyc portion which is essential for the catalytic activity. For **2**, the contaminants do not contain some of the nucleobases which are important for complex formation with Mb. It was assumed, therefore, that the presence of small amounts of the contaminants do not affect the catalytic outcome significantly.

Up to 2.5 or 6 molecules of Mb were cleaved by each molecule of Cu(II)**1** or Co(III)**1**, respectively, for the data of Figure 2, indicating the catalytic nature of the action of Cu(II)**1** and Co(III)**1**. 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)**1**, in spite of 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.[29]

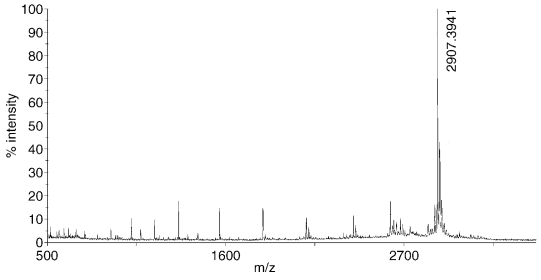


Figure 8. MALDI-TOF MS of Co(III)**1**; m/z 2907.39 $(M+H)^+$ ($C_{111}H_{153}N_{64}O_{25}S_2Co$ calcd 2906.91).

The rate for cleavage of Mb by Cu(II)**1** or Co(III)**1** was unaffected by the removal of O_2 from the reaction mixtures. This result and previous observation[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 the catalysts examined in this study. The hydrolytic nature of the Mb cleavage was further supported by the successful N-terminal sequencing of the cleavage products obtained with Co(III)**3** by Edman degradation, which demonstrates generation of a primary amino group by the Mb cleavage.

The lack of catalytic activity of Co(III)**18** in Mb cleavage in contrast to Co(III)**1** indicates that the sequence of the PNA oligomer contained in **1** is important for the catalytic activity, in view of the subtle difference in the structure of **1** and **18**. The Cu(II) complex of **1** as well as the Co(III) complexes of **1**, **3**, **6**, **7**, **9**, and **10** can be regarded as artificial proteases selective for Mb on the basis of the catalytic turnover illustrated in Figure 2, hydrolytic cleavage of the peptide bonds, and recognition of Mb.

Among the metal ions tested, Co(III) manifested the highest protein-cleaving activity upon complexation to **1**. 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 due to the exchange-inertness of Co(III). The k_{cat} measured with Co(III)**1** at pH 7.5 and 37 °C corresponds to the half-life of 30 h. This may be compared with the half-life[30] of 200 years for spontaneous hydrolysis of unactivated amides measured under identical conditions. The k_{cat} may be further compared with the k_{cat} of 0.18 h^{-1} (at the optimum pH of 9 and 25 °C; corresponding to a half-life of 3.8 h) measured with a catalytic antibody[31] with peptidase activity elicited by a joint hybridoma and combinatorial antibody library approach in the hydrolysis of an amide substrate. Both Co(III)**1** and the catalytic antibody manifest high affinity for their substrates. The k_{cat} of the Mb-selective artificial protease can be improved further by raising the effective molarity of the catalytic group toward the scissile peptide linkage in the complex formed between Mb and the artificial protease.

Mb is cleaved at two different positions by the action of Co(III)**1** producing two pairs of protein fragments. It is not clear at present whether the two cleavage sites involve different Co(III)**1**–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. Since it was unsuccessful to identify the N-terminal residues of the cleavage products obtained with Co(III)**1**, analogues of Co(III)**1** containing both the PNA moiety and the Co(III)Cyc unit were synthesized. A change in the relative position of the PNA portion and the Co(III)Cyc would induce different catalytic behavior, and, thus, Mb might be cleaved at one site, allowing identification of the newly formed N-terminal amino acid residue. Indeed, the analogues cleaved Mb at one position as summarized in Table 2 and N-terminal sequencing experiment performed with the cleavage products obtained with Co(III)**3** clearly identified the cleavage site.

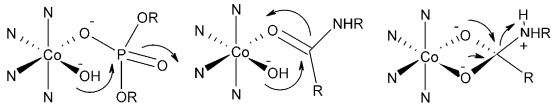
The PNA 9-mer contained in **1** is presumably bound on the surface of Mb, although no information is available at present for the structure of the complex formed between the PNA 9-mer and Mb. It would be possible to design protein-cleaving catalysts by attaching the catalytic center either to the N- or the C-terminus of the PNA portion of **1** if both of the termini are located on the surface of Mb in the PNA–Mb complex. In Co(III)**2**, the Co(III)Cyc is attached to the C-terminus. With Co(III)**2**, however, Mb was not cleaved. It is possible that Co(III)Cyc is not located in a productive position in the Co(III)**2**-Mb complex. It is also possible that the L-Lys moiety of Co(III)**1** is essential for recognition of Mb and that Co(III)**2** is inactive due to the absence of the L-Lys moiety.

In Co(III)**3**, the methyl group was attached to the Cyc-acetyl moiety in an attempt to suppress rotational freedom of the acetyl moiety connecting Cyc to the binding site. The catalytic activity, however, was considerably reduced. The additional methyl group did not raise the effective molarity of the catalytic center in the Mb–catalyst complex. In Co(III)**4**–Co(III)**8**, an amino acid moiety is inserted between the Cyc-acetyl moiety and the binding site. When Gly or β-Ala was inserted [Co(III)**4** or Co(III)**8**], the catalytic activity was almost completely lost. Elongation of the linker by three or four atoms could raise the rotational freedom of the linker, reducing the effective molarity of Co(III)Cyc in the catalyst–Mb complex. When L-Asp was inserted [Co(III)**5**], the catalytic activity was lost again. The extra carboxyl group introduced by Asp did not produce an active conformation for the catalyst. On the other hand, catalytic activity was manifested when L-Pro or L-Lys [Co(III)**6** or Co(III)**7**] was inserted. The unique conformation of L-Pro apparently provided the catalyst with productive conformation. The cationic center introduced to the linker by the ε-amino group of L-Lys in Co(III)**7** provided a productive conformation presumably by electrostatic interaction with the Mb surface. It is noteworthy that Mb is cleaved at the same position even when the linker was elongated by three atoms in Co(III)**6** and Co(III)**7** compared with Co(III)**1** and Co(III)**3**. In Co(III)**9**, an additional Co(III)Cyc moiety is introduced to the ε-amino group of the L-Lys moiety of Co(III)**7**. The catalytic activity, however, was not considerably improved upon introduction of the additional Co(III)Cyc moiety. Since identical protein fragments were obtained for Co(III)**7** and Co(III)**9**, it appears that the Co(III)Cyc attached to the α-amino group, rather than the ε-amino group, of the L-Lys portion in Co(III)**9** is the catalytic group.

Metal ions can play several catalytic roles in hydrolysis of peptide bonds.[32,33] Metal ion itself can act as a Lewis acid to enhance the electrophilicity of the carbonyl carbon of the scissile bond upon coordination to

the carbonyl oxygen. Metal-bound hydroxide ion can act as a nucleophile that attacks at the carbonyl carbon of the peptide bond. In addition, the metal-bound water molecule can act as a general acid to protonate the leaving nitrogen in the rate-determining expulsion of the amine from the tetrahedral intermediate. Sometimes, the metal ion can play several catalytic roles simultaneously.

The Co(III) complexes of Cyc and related tetraaza ligands catalyze the hydrolysis of phosphodiesters including DNA.[34–40] The mechanism of **19** has been suggested for the phosphodiester hydrolysis.[34,36] Protein-cleavage by Co(III)Cyc of the Mb-cleaving catalysts involves hydrolysis of peptide bonds. A mechanism (**20**) similar to **19** can be proposed for the peptide hydrolysis by Co(III)**1** or its analogues. An analogous mechanism involving formation of a four-membered ring has been proposed for metal-catalyzed amide hydrolysis.[34,41] The rate-determining step for the mechanism of **20** would be expulsion of the amine moiety from the tetrahedral intermediate (**21**), which requires the protonation of the leaving amine. The bell-shaped pH profiles of k_{cat} illustrated in Figures 4 and 5 agree with the mechanism of **20**/**21**, since protonation of the hydroxo (**20**) or oxo (**21**) ligand at low pHs and the deprotonation of the ammonium ion (**21**) at high pHs would reduce the reactivity.



19 **20** **21**

The effectiveness of protein-cleaving catalytic drugs exploiting metal complexes as the catalytic centers would depend on the structure of the chelating ligands of the catalytic centers. In this regard, various chelating ligands were tested in the present study. Compounds **1** and **10–14** have characteristic structural features: **1** contains a macrocyclic tetraaza ligand, **10** contains a macrocyclic triaza-monooxo ligand, **11** contains a macrocyclic triaza ligand, **12** contains an acyclic tetraaza ligand with one aromatic nitrogen atom, **13** contains an acyclic triaza ligand with one aromatic nitrogen atom, and **14** contains an aliphatic acyclic tetraaza ligand. How the catalytic outcome is affected by the chelating ligands with the various structural features was examined.

MALDI-TOF MS of the product solutions revealed that an identical peptide bond of Mb is cleaved by Co(III)**1** and Co(III)**10**. Co(III)**10** complexed to Mb is 2–3 times less reactive than Co(III)**1** bound to Mb as revealed by the k_{cat} values summarized in Table 1. On the other hand, Co(III) complexes of **11–14** did not show any catalytic activity to cleave Mb, although the PNA portions of the complexes should have been bound to Mb. Marked differences are observed for the catalytic activities of Co(III) complexes of **1** and **10–14**, demonstrating that selection of the chelating ligand for the metal center is important in the design of the protein-cleaving catalytic drugs.

In the complex formed between the Co(III) complexes and Mb, the distance between the Co(III) center and the carbonyl group of the target amide bond would be affected by the nature of the chelating ligand. Thus, the Co(III) ion chelated by **1** may occupy the most productive position among various chelating ligands examined here. A small difference in the distance between the catalytic center and the reaction site can cause a dramatic change in catalytic rates.[18] If the marked difference seen with the Co(III) complexes of **1** and **10–14** is due to this effect, relative activities of **1** and **10–14** would be different for individual catalytic drugs targeted to various proteins.

The intrinsic catalytic power of the Co(III) center should be controlled by the structure of the ligand. The Lewis acidity of Co(III) would depend on the nature of the ligating atoms as well as relative positions of the ligating atoms. In addition, the steric strain involved in the rate-determining transition state should be affected by the structure of the chelating ligands. For the phosphodiester hydrolysis by Co(III) complexes of tetraaza ligands (**19**), for example, the catalytic rate was sensitive to the structure of the chelating ligands. This was attributed to the stabilization of the four-membered ring in the transition state,[34,35] since the steric strain in the ring should be affected by the structure of the chelating ligands of the Co(III) center. Similarly, the steric strain of the four-membered ring of mechanism **20** should depend on the structure of the chelating ligands. If the marked difference seen with the Co(III) complexes of **1** and **10–14** is due to the intrinsic reactivity of the Co(III) center originating from electronic and/or steric effects of the ligands, Cyc is better than **10–17** as the chelating ligand for protein-cleaving catalytic drugs.

The catalytic center and the binding site are the two essential components of target-selective artificial proteases. A large number of small molecules are known to have high affinity for various disease-related proteins. Those molecules can be employed as the binding sites of the artificial proteases. Small molecules with high affinity for a target enzyme or receptor may be searched by the combinatorial method. Even if they have high affinity, they cannot be used as drugs unless the target proteins are considerably deactivated upon complexation with the small molecules. Those molecules, however, can be used as effective binding sites for the artificial proteases selective for target proteins.

The present study demonstrates that target-selective artificial proteases can be designed by choosing appropriate binding sites, linkers, and catalytic units. The activity of a target-selective artificial protease 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. If the targets are disease-related proteins, the artificial proteases can serve as protein-cleaving catalytic drugs.

Experimental

Synthesis of catalysts

PNA derivatives were synthesized by automated procedures using an Expedite Model 8909 Nucleic Acid Synthesis System with various building blocks. Building blocks **GX**, **CX**, **GlyX**, **AspX**, **ProX**, **BalX**, and **LysX** were purchased from commercial sources whereas **A*X** and **T*X** as well as **1X** were synthesized as reported[20] previously and **2Y**, **3X**, and **9X**–**17X** were synthesized as summarized in Schemes 1–11. **2Y**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72 (m, 2H), 7.57 (m, 2H), 7.40–7.16 (m, 19H), 5.05–4.85 (br s, 6H), 4.37 (m, 1H), 4.20–4.18 (m, 2H), 4.06–3.95 (br s, 4H), 3.70 (br s, 2H), 3.40–3.10 (br m, 18H), 2.83–2.78 (br s, 4H); HRMS exact mass 1013.1403 $(M+H)^+$, calcd for $C_{55}H_{62}N_7O_{12}$ 1013.1370. **3X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.53–3.30 (br, 14H), 2.96 (br s, 4H), 1.43 (m, 27H); MS (MALDI-TOF) *m*/*z* 531.75 $(M+H)^+$ ($C_{25}H_{47}N_4O_8$ calcd 531.67). **9X**: ^{1}H NMR (300 MHz, CD_3OD) 4.14 (m, 1H), 3.17–3.46 (br m, 28H), 3.14 (t, 2H), 2.79–2.70 (br m, 8H), 1.73 (m, 1H), 1.55 (m, 1H), 1.36 (m, 54H), 1.33–1.20 (m, 4H); MS (MALDI-TOF) *m*/*z* 1172.48 $(M+H)^+$ ($C_{56}H_{103}N_{10}O_{16}$ calcd 1172.49). **10X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34–7.25 (m, 10H), 5.11 (m, 4H), 4.23–4.08 (br m, 4H), 3.84–3.50 (br m, 10H), 3.25–3.15 (br m, 4H); MS (MALDI-TOF) *m*/*z* 500.50 $(M+H)^+$ ($C_{26}H_{34}N_3O_7$ calcd 500.58). **11X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34 (m, 10H), 5.15 (d, 4H), 3.38 (m, 10H), 2.74 (br s, 4H); MS (MALDI-TOF) *m*/*z* 456.06 $(M+H)^+$ ($C_{24}H_{30}N_3O_6$ calcd 456.52). **12X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63 (t, 1H), 7.14 (br d, 2H), 5.85 (br s, 1H), 4.54 (s, 2H), 4.39 (s, 2H), 3.81 (br s, 2H) 3.45 (br s, 4H), 1.45 (m, 27H); MS (MALDI-TOF) *m*/*z* 539.75 $(M+H)^+$ ($C_{26}H_{43}N_4O_8$ calcd 539.65). **13X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63 (t, 1H), 7.28 (d, 1H), 7.18 (d, 1H), 5.45 (br s, 1H), 4.58 (s, 2H), 4,40 (d, 2H), 4.16 (s, 2H), 1.50 (m, 18H); MS (MALDI-TOF) *m*/*z* 396.52 $(M+H)^+$ ($C_{19}H_{30}N_3O_6$ calcd 396.47). **14X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.98 (s, 2H), 3.32–3.16 (m, 6H), 2.65 (br s, 6H), 1.45 (m, 27H); MS (MALDI-TOF) *m*/*z* 505.74 $(M+H)^+$ ($C_{23}H_{45}N_4O_8$ calcd 505.64). **15X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.18 (br s, 2H), 3.23–3.18 (m, 6H), 2.76–2.70 (m, 4H), 1.46 (s, 18H); MS (MALDI-TOF) *m*/*z* 362.53 $(M+H)^+$ ($C_{16}H_{32}N_3O_6$ calcd 362.44). **16X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.35–5.25 (br d,1H), 3.90 (d, 2H), 3.39 (m, 2H), 3.20 (m, 2H), 1.47–1.42 (m, 18H); MS (MADLI-TOF) *m*/*z* 319.42 $(M+H)^+$ ($C_{14}H_{27}N_2O_6$ calcd 319.38). **17X**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.45 (s, 4H), 2.78 (t, 2H), 2.50 (t, 2H), 1.79 (dd, 2H), 1.46 (s, 18H); MS (MALDI-TOF) *m*/*z* 332.46 $(M+H)^+$ ($C_{16}H_{30}N_1O_6$ calcd 332.42).

The protocol of the commercial automated system can be summarized as follows: The solid support was a Sieber amide resin built on crosslinked polystyrene onto which polyethyleneglycol was grafted. The amino group generated on treatment of the resin with trifluoroacetic acid (TFA) was coupled with the carboxyl group of a building block with the aid of *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. The resulting resin was treated with acetic anhydride to block unreacted amino group. By treatment with piperidine, fmoc group of the newly introduced building block was removed to generate an amino group which was used for further coupling with the carboxyl group of another building block. The PNA oligomer derivatives were detached from the solid support by treatment with TFA and cresol, and were precipitated by addition of cold ether. The carboxy group attached to the resin in the first coupling step was released as a carboxamide. Then, the protecting groups attached to the nucleobases and chelating ligands were removed with TFA and trifluoromethanesulfonic acid. In the synthesis of the library of $CycAc(Q)_nLysNH_2$ by the Split-Pool method, equal amounts of **A*X**, **T*X**, **GX**, and **CX** were used in the reaction mixture employed in coupling with the amino ends attached to the polymer support. The same general procedure was used for the synthesis of **1**–**18**. MS (MALDI-TOF) *m*/*z* 2851.49 $(M+H)^+$ ($C_{111}H_{154}N_{64}O_{25}S_2$ calcd 2848.97) for **1**, 2879.63 $(M+H)^+$ ($C_{111}H_{154}N_{66}O_{25}S_2$ calcd 2876.99) for **2**, 2864.43 $(M+H)^+$ ($C_{112}H_{156}N_{64}O_{25}S_2$ calcd 2863.00) for **3**, 2906.46 $(M+H)^+$ ($C_{113}H_{157}N_{65}O_{26}S_2$ calcd 2906.21) for **4**, 2964.86 $(M+H)^+$ ($C_{115}H_{159}N_{65}O_{28}S_2$ calcd 2964.06) for **5**, 2945.94 $(M+H)^+$ ($C_{116}H_{161}N_{65}O_{26}S_2$ calcd 2946.08) for **6**, 2977.85 $(M+H)^+$ ($C_{117}H_{166}N_{66}O_{26}S_2$ calcd 2977.14) for **7**, 2920.36 $(M+H)^+$ ($C_{114}H_{159}N_{65}O_{26}S_2$ calcd 2920.05) for **8**, 3191.87 $(M+H)^+$ ($C_{127}H_{186}N_{70}O_{27}S_2$ calcd 3189.43) for **9**, 2851.49 $(M+H)^+$ ($C_{111}H_{153}N_{63}O_{26}S_2$ calcd 2849.85) for **10**, 2807.51 $(M+H)^+$ ($C_{109}H_{149}N_{63}O_{25}S_2$ calcd 2805.90) for **11**, 2858.87 $(M+H)^+$ ($C_{112}H_{150}N_{64}O_{25}S_2$ calcd 2856.95) for **12**, 2816.37 $(M+H)^+$ ($C_{110}H_{145}N_{63}O_{25}S_2$ calcd 2813.88) for **13**, 2824.04 $(M+H)^+$ ($C_{109}H_{152}N_{64}O_{25}S_2$ calcd 2822.93) for **14**, 2781.94 $(M+H)^+$ ($C_{107}H_{147}N_{63}O_{25}S_2$ calcd 2779.87) for **15**, 2739.11 $(M+H)^+$ ($C_{105}H_{142}N_{62}O_{25}S_2$ calcd 2736.80) for **16**, 2839.36 $(M+H)^+$ ($C_{109}H_{145}N_{61}O_{29}S_2$ calcd 2837.86) for **17**, 2824.08 $(M+H)^+$ ($C_{111}H_{155}N_{61}O_{26}S_2$ calcd 2823.96) for **18**.

Quantification of PNA derivatives was carried out with UV spectra by approximating that the absorbance at 260 nm of a PNA derivative was equal to the sum of those of constituent nucleobases. Insertion of Co(III) ion to the chelating ligands of **1**–**18** was carried out according the literature method by insertion of Co(II) ion followed by air-oxidation of the Co(II) ion to Co(III).[27]

Measurements

In kinetic measurements, the temperature was controlled with an immersion circulator manufactured by Fisher Scientific. 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. Distilled and deionized water was used for preparation of buffer solutions. Buffers (0.05 M) used in this study were acetate (pH 4.5–5.0), 2-(*N*-morpholino)ethanesulfonate (pH 5.5–6.5), *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonate (pH 7–8), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (pH 8.5) and boric acid (pH 9). All buffer solutions were filtered with 0.45 μm Millipore microfilter and autoclaved before use in the kinetic measurements. The stock solution of Mb was prepared by dissolving horse heart Mb (purchased from Sigma, used without further purification) in water and was kept at 4 °C. Mb is oxidized to metMb in the presence of oxygen. The Mb used in the present study was also in the met form as checked by its Vis spectrum.[42]

Acknowledgements

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