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Artificial Metalloprotease Based on Co(III)oxacyclen-Aldehyde Conjugate†

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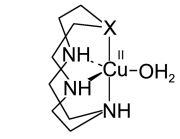
Proteases share about 60% of the total worldwide sale of industrial enzymes.[1] Most of the industrial proteases have broad substrate selectivity, hydrolyzing a variety of protein substrates they encounter. In view of thermal and chemical instabilities of natural enzymes, synthesis of protease-like catalysts (artificial proteases) with broad substrate selectivity and high thermal and chemical stabilities is important.

The Cu(II) and Co(III) complexes of 1,4,7,10-tetraazadodecane (cyclen) have been often exploited as the catalytic centers for artificial proteases.[2-11] Proteolytic activity of the Cu(II) complex (Cu(II)cyclen) of cyclen was greatly enhanced on attachment to polystyrene backbone apparently due to the hydrophobic microenvironment.[3] When the aldehyde group was positioned in proximity of the Cu(II)cyclen anchored on crosslinked polystyrene, heterogeneous artificial proteases with high proteolytic activity and broad substrate selectivity were produced.[8] This was due to reversible imine formation between the aldehyde group of the artificial protease and the ammonium ion exposed on the surface of the substrate protein. A later study disclosed that the Cu(II) complex (Cu(II)oxacyclen) of 1-oxo-4,7,10-triazadodecane (oxacyclen) had a much higher proteolytic activity compared with Cu(II) cyclen owing to the enhanced Lewis acidity of the Cu(II) center.[12] Introduction of the aldehyde group in proximity to Cu(II)oxacyclen produced homogeneous artificial proteases with high proteolytic activity, broad substrate selectivity, and high thermal and chemical stabilities.[13] The activity of the homogeneous artificial protease based on the Cu(II)oxacyclen-aldehyde conjugate was comparable to the heterogeneous artificial protease based on the Cu(II)cyclen-aldehyde-polystyrene conjugate.

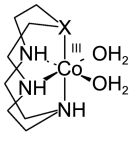
Artificial proteases based on the exchange-inert[14] Co(III) complexes have advantages compared with those on the exchange-labile Cu(II) complexes in practical applications. Abstraction of the metal centers from the catalysts by metal-binding substances present in the reaction mixture may limit the lifetime of the catalyst. It was reported that the proteolytic activity of the Co(III) complex (Co(III)oxacyclen) of oxacyclen is much higher than that of the Co(III) complex (Co(III) cyclen) of cyclen due to the enhanced Lewis acidity of the Co(III) center.[15] In the present study, a homogeneous artificial protease based on the Co(III)oxacyclen-aldehyde conjugate was synthesized and its proteolytic activity was characterized.

As the aldehyde-containing artificial protease, Co(III)**A**, the Co(III) complex of 4-((4-(2-methyl-butylamino)-6-(3-(1-oxa-4,7,10-triaza-cyclododec-7-yl)-propylami-no)-1,3,5-triazin-2-ylamino)-methyl)-benzaldehyde (**A**), was synthesized. As the control of Co(III)**A**, the Co(III) complex (Co(III)**B**) of *N*-benzyl-*N*'-(2-methyl-butyl)-*N*''-(2-(1-oxa-4,7,10-triaza-cyclododec-7-yl)-ethyl)-1,3,5-triazine-2,4,6-triamine (**B**), was prepared. As the protein substrates, bovine serum albumin (Alb), bovine serum γ-globulin (Glo), horse heart myoglobin (Myo), and chicken egg white lysozyme (Lys) were employed. Myoglobin is oxidized to metmyoglobin in the presence of oxygen and the myoglobin used in the present study was also in the met form as checked by its visible spectrum.[16]

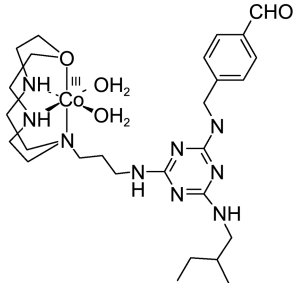
The rates for disappearance of the protein substrate during the reaction with Co(III)**A** or Co(III)**B** was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis,[17] as described previously.[3,6,8,12,13,15] The pseudo-first-order kinetic constant (k_0) thus obtained is related to the cleavage of the substrate protein molecule itself and does not provide information on further fragmentation of the initial cleavage products. Glo has two subunits with distinctly different molecular weights (25 and 50 kDa). Since the density of the electrophoretic band of the light chain was weak, the kinetic



Cu(II)cyclen: X=NH
Cu(II)oxacyclen: X=O



Co(III)cyclen: X=NH
Co(III)oxacyclen: X=O



Co(III)**A**: Y=CHO
Co(III)**B**: Y=H

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.

data were collected only for the degradation of the heavy chain.

The k_0 values were measured for each substrate in the presence of Co(III)**A** at various pH's and at fixed C_0 (initially added catalyst concentration) and S_0 (initially added substrate concentration) under the conditions of C_0 (0.2 mM) >> S_0 (3-10 μM) to identify the optimum pH. The kinetic data were obtained up to pH 9.5 to avoid complications due to background alkaline degradation of proteins at higher pH's. At the optimum pH thus selected, the k_0 values were measured at various C_0 concentrations under the conditions of C_0 >> S_0. As shown by the dependence of k_0 on C_0 (Figs. 1-4), saturation kinetic behavior was observed for the cleavage of the protein substrates by Co(III)**A** and Co(III)**B**.

Kinetic data for the cleavage of the protein substrates by Co(III)**A** or Co(III)**B** were analyzed in terms of Michaelis-Menten scheme (eq. 1). Under the conditions of C_o >> [*CS*], pseudo-first-order kinetic behavior is expected with k_0 being derived as eqs. 2-4. Here, [*CS*] is the steady state concentration of *CS*. The dependence of k_0 on C_0 was analyzed to estimate k_{cat}, K_m, and their standard deviations. The values of k_{cat} and K_m thus estimated for the cleavage of the protein substrates by Co(III)**A** or Co(III)**B** are summarized in Table 1.

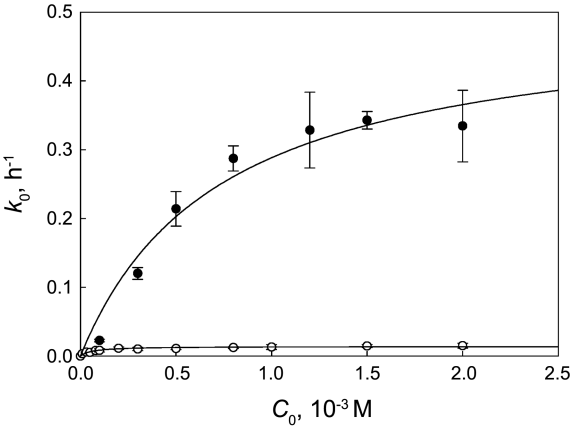


Figure 1. Plot of k_0 against C_0 for cleavage of Alb (3.0 μM) by Co(III)**A** (filled circle) or Co(III)**B** (open circle) at pH 9.5 and 50 °C.

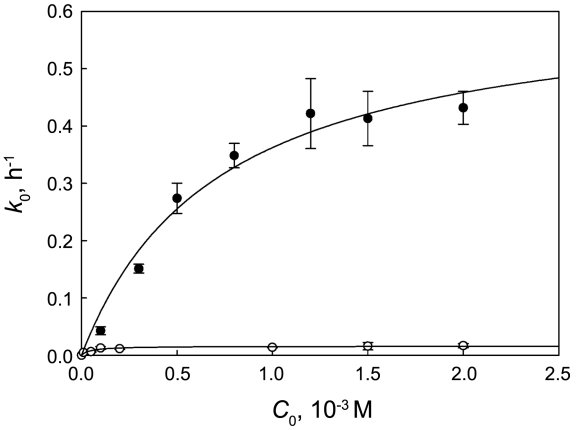


Figure 2. Plot of k_0 against C_0 for cleavage of Glo (2.7 μM) by Co(III)**A** (filled circle) or Co(III)**B** (open circle) at pH 9.5 and 50 °C.

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

$$k_o = k_{cat}C_0/(K_m + C_0) \quad (2)$$

$$k_{cat} = k_2 \quad (3)$$

$$K_m = (k_{-1} + k_2)/k_1 = [C][S]/[CS] \quad (4)$$

The results summarized in Figs. 1-4 indicate that the proteolytic activity of the Co(III) center is considerably en-

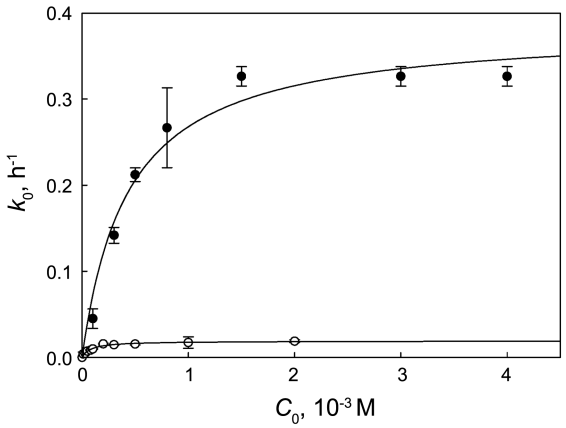


Figure 3. Plot of k_0 against C_0 for cleavage of Myo (10 μM) by Co(III)**A** (filled circle) or Co(III)**B** (open circle) at pH 8.0 and 50 °C.

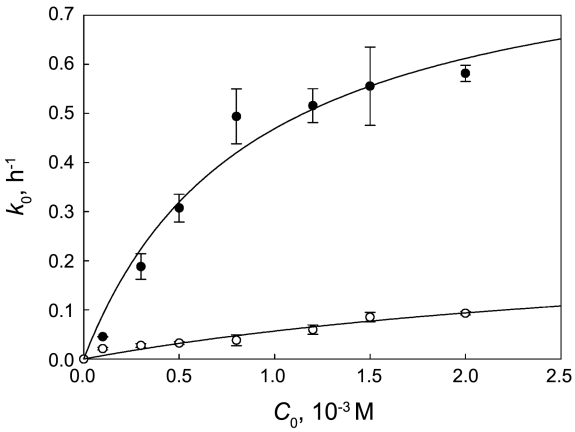


Figure 4. Plot of k_0 against C_0 for cleavage of Lys (7.0 μM) by Co(III)**A** (filled circle) or Co(III)**B** (open circle) at pH 9.5 and 50 °C.

Table 1. Values of kinetic parameters for the cleavage of various protein substrates by Co(III)**A**, and Co(III)**B** at 50 °C

substrate	catalyst	pH	k_{cat} (h^{-1})	K_m (10^{-4} M)
Alb	Co(III)**A**	9.5	0.62 ± 0.06	7.2 ± 1.4
	Co(III)**B**	9.5	0.016 ± 0.001	0.47 ± 0.14
Glo	Co(III)**A**	9.5	0.50 ± 0.06	7.3 ± 1.7
	Co(III)**B**	9.5	0.014 ± 0.001	0.64 ± 0.10
Myo	Co(III)**A**	8.0	0.40 ± 0.02	4.8 ± 0.6
	Co(III)**B**	8.0	0.019 ± 0.002	0.84 ± 0.11
Lys	Co(III)**A**	9.5	0.88 ± 0.11	9.0 ± 3.0
	Co(III)**B**	9.5	0.27 ± 0.15	37 ± 22

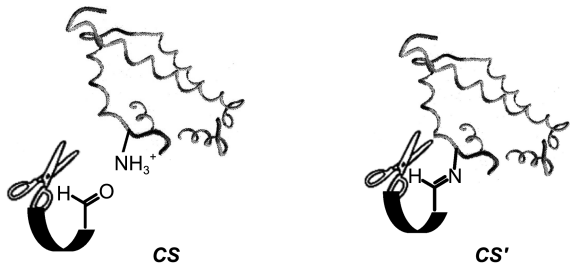


Table 2. Effect of $Na(CN)BH_3$ on the rate constant in cleavage of 3.0 μM albumin by 1.0 mM Co(III)**A** and Co(III)**B** at pH 6.0 and 50 °C

catalyst	[$Na(CN)BH_3$] (mM)	k_0 (h^{-1})
Co(III)**A**	0	0.030 ± 0.003
	5.0	0.42 ± 0.03
Co(III)**B**	0	0.0097 ± 0.0023
	5.0	0.0095 ± 0.0021

hanced by incorporation of the aldehyde group in proximity at all of the C_0 concentrations examined. This may be attributed to the reversible formation of the imine bond between the aldehyde group of the catalyst and the ammonium group of the substrate in the catalyst-substrate complex (*CS*), leading to the formation of the covalent intermediate (*CS*'), as proposed in the previous studies.[8,13]

To obtain evidence for the existence of the imine-Co(III)**A** catalysts, it was attempted to trap the imine with $Na(CN)BH_3$, the reducing agent selective for imines in water,[13,18] and the results are summarized in Table 2. If the imine bond is reduced to the amine bond, reverse reaction of *CS*' to form *CS* is blocked, leading to rate enhancement for cleavage of the protein substrate.[13] The results summarized in Table 2 indicate that $Na(CN)BH_3$ raises the proteolytic rate of Co(III)**A** without affecting the rate for Co(III)**B**. The results obtained with Na $(CN)BH_3$ support the existence of an imine intermediate in the proteolytic action of Co(III)**A**.

Examination of the results summarized in Table 1 discloses that the faster rates of Co(III)**A** compared with Co(III)**B** originate from the greater values of k_{cat}. The values of K_m are, however, more favorable for Co(III)**B** except for Lys. The Michaelis-Menten scheme of eq. 1 can be modified as eq. 5 to include *CS*'. The same saturation kinetic behavior (eqs. 2 and 6) is predicted for the dependence of k_0 on C_0 by both eq. 1 and eq. 5. By applying the steady-state approximation to *CS* and *CS*', eqs. 7 and 8 are derived. Here, [*CS*] and [*CS*'] are the steady state concentrations of *CS* and *CS*', respectively.

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CS' \xrightarrow{k_3} C + P_1 + P_2 \quad (5)$$

$$k_o = k_{cat}^{app} C_0/(K_m^{app} + C_0) \quad (6)$$

$$k_{cat}^{app} = k_2k_3/(k_2 + k_{-2} + k_3) = k_3/(1 + [CS]/[CS']) \quad (7)$$

$$K_m^{app} = (k_2k_3 + k_{-1}k_3 + k_{-1}k_{-2})/k_1(k_2 + k_{-2} + k_3) = [C][S]/([CS] + [CS']) \quad (8)$$

The higher values of k_{cat} (or k_{cat}^{app}) of Co(III)**A** compared with those of Co(III)**B** (Table 1) are consistent with the higher effective molarity of the catalytic center toward the scissile peptide bond in *CS*' owing to more favorable entropic factor. Eq. 7 indicates that the limiting value of k_{cat}^{app} is k_3, the first-order rate constant for the reaction of the Co(III) center with the scissile bond in *CS*'. If [*CS*'] is smaller than [*CS*], however, k_{cat}^{app} is smaller than k_3, reducing the catalytic efficiency at high C_0 concentrations.

The higher values of K_m (or K_m^{app}) of Co(III)**A** compared with those of Co(III)**B** (Table 1) indicate that the sum of [*CS*] and [*CS*'] for Co(III)**A** is smaller than [*CS*] for Co(III)**B** when the same values of C_0 are used. This reveals that *CS*' does not accumulate in a significant amount.

The data reported in the previously study for the Cu(II) complex of **A** (Cu(II)**A**) and the Cu(II) complex of **B** (Cu(II)**B**) showed that k_{cat} is raised and K_m is lowered, leading to the improvement in both k_{cat} and K_m upon introduction of the aldehyde group in the vicinity of the Cu(II) center. In the action of Cu(II)**A**, *CS*' appears to accumulate in a significant amount in contrast with Co(III)**A**. The values of k_{cat} and K_m for the action of Cu(II)**A** for the proteins listed in Table 1 were 0.2-0.8 h^{-1} and 0.5-5 mM, respectively. Although the values of K_m were more favorable for Cu(II)**A** compared with Co(III)**A**, similar values of k_{cat} were obtained for Cu(II)**A** and Co(III)**A**.

In conclusion, the proteolytic activity of Co(III)oxacyclen was considerably improved by conjugation with the aldehyde group owing to formation of covalent intermediates with protein substrates. The proteolytic activity of Co(III)oxacyclen-aldehyde conjugate was comparable to that of Cu(II)oxacyclen-aldehyde conjugate. The Co(III)-based homogeneous artificial proteases would have advantages compared with the Cu(II) analogues due to the exchange inertness of the metal center.

Experimental Section

Synthesis of **A** and **B** was described in the literature.[13] The Co(III) complexes of **A** and **B** were prepared according to the method reported previously.[7,9-11,15] The substrate proteins were obtained from Sigma. Rate measurements carried out as reported previously.[13]

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