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Cooperation of β-Cyclodextrin with Macrocyclic Metal Centers in the Action of Artificial Metalloesterases Built on Poly(ethylenimine)

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β-Cyclodextrin (CD) was attached to poly(ethylenimine) (PEI), and then macrocyclic metal centers were constructed on the polymer by condensation with glyoxal or butanedione in the presence of metal (Zn^{II}, Ni^{II}, or Co^{II}) templates. The resulting polymers were treated with acetic anhydride to block primary or secondary amines. Hydrolysis of two nitrophenyl esters containing *t*-butylphenyl residues in the acyl moieties was catalyzed by the PEI derivatives containing both CD cavities and macrocyclic metal centers. Analysis of the kinetic data indicated that the CD cavities and the metal centers provided binding sites and catalytic sites, respectively, for the ester substrates, mimicking metalloesterases. Although the content of metal center was much smaller for the Zn^{II}-containing artificial enzyme compared with the Ni^{II} and Co^{II} analogs, catalytic efficiency was greater for the Zn^{II} derivative. Kinetic data indicate high reactivity of the metal center in the productive complex formed between the catalyst and the substrate, when the unproductive binding by the CD cavities is considered.

INTRODUCTION

Although a large number of synthetic compounds have been designed as biomimetic host molecules (*1–8*), characteristics of enzymatic action have rarely been reproduced with the host compounds. Molecular recognition of a substrate is necessary for complexation with the substrate. In addition, more effective molecular recognition of the transition state is required to achieve high-rate acceleration.

Many cyclodextrin derivatives have been examined for their ability to recognize guest molecules and to catalyze chemical transformation of the included molecules (*7–11*). In order to devise effective biomimetic catalysts using cyclodextrins, it is necessary to introduce catalytic groups to cyclodextrins in positions suitable for high catalytic efficiency.

Artificial enzymes are synthetic catalysts manifesting characteristics of enzymatic actions such as complex formation with substrates, high-rate acceleration, and high selectivity with respect to the substrate structures or chemical transformations involved. In the design of artificial enzymes, it is necessary to create

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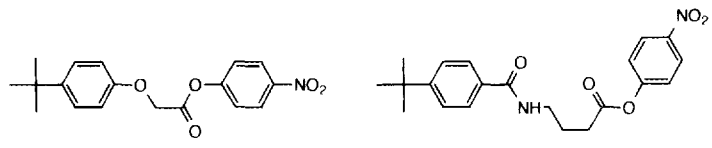


1 2

SCHEMES 1 AND 2

active sites which accommodate several catalytic elements. Poly(ethylenimine) (PEI) has been exploited as a molecular backbone for such active sites, due to its water solubility, easy modification through alkylation, acylation, or imine-formation of the amino groups, and highly branched structure (*11–20*). Recently, we have attached β-cyclodextrin (CD) to PEI, obtaining a PEI derivative with specific binding sites as well as a CD derivative with convergent amino groups located above the CD cavity (*11*). We have also constructed macrocyclic metal centers on PEI by condensation of PEI with dicarbonyl compounds in the presence of metal templates (*15*).

In our next step toward the design of effective artificial enzymes based on PEI equipped with CD as the binding site, introduction of catalytic groups to the polymer backbone in the vicinity of the CD cavity is attempted. Since many catalytic roles are played by metal ions acting as Lewis acid catalysts in various organic reactions (*13*, *14*), metal ions are chosen as catalytic groups to cooperate with the CD binding sites. In this paper, preparation of PEI derivatives equipped with both macrocyclic metal centers and CD cavities is described, together with the analysis of their activity as artificial metalloesterases in the hydrolysis of 4-nitrophenyl (4-*t*-butylphenoxy)acetate (**1**) and 4-nitrophenyl *N*-(4-*t*-butylbenzoyl)-4-aminobutyrate (**2**) (Schemes 1 and 2).

EXPERIMENTAL PROCEDURES

PEI Derivatives

PEI (MW 50,000) was purchased from Sigma and purified by ultrafiltration with a PM-30 membrane (Amicon) or by dialysis to remove portions of low molecular weight, and the average molecular weight of the purified PEI was estimated as ca. 60, 000. Attachment of CD to PEI was carried out as reported previously (*11*), producing CD_1–PEI in which the molar content of the CD moiety was 1.4% of the monomer residues of PEI.[2] [CD_1–PEI]Ac was prepared by acetylation of CD_1–PEI with excess acetic anhydride according to the general procedure reported in the literature (*21*).

[2] The subscripts included in the abbreviated names of the PEI derivatives represent the percentage molar contents of inorganic or organic modifiers relative to monomer residues.

Ni^{II}_1[CD_1–PEI–GO] was prepared through the Ni^{II}-template condensation of CD_1–PEI with glyoxal according to the general procedure (*17*) described previously for Ni^{II}_7[PEI–GO] by using glyoxal (40% solution, 1.30 g; 22.4 mmol), $NiCl_2 \cdot 6H_2O$ (8.57 g; 36.1 mmol) and CD_1–PEI (0.28 M, 200 ml; 56 residue mmol). Lyophilization of the polymer produced light-brown powders and the metal content of the polymer was 1.0% of the monomer residues.

Ni^{II}_1[CD_1–PEI–GO]Ac was prepared according to the general procedure reported in the literature (*21*) by acetylation of Ni^{II}_1[CD_1–PEI–GO] (300 ml, 0.19 residue M) with excess acetic anhydride, and purified by dialysis against water (12 liters), 0.1 M NaCl (12 liters × 3 times), and water (12 liters × 3 times).

$Zn^{II}_{0.2}$[CD_1–PEI–GO] was prepared through the Zn^{II}-template condensation of CD_1–PEI with glyoxal according to the method described above for Ni^{II}_1 [CD_1–PEI–GO] by using glyoxal (40% solution, 1.30 g; 22.4 mmol), $ZnCl_2$ (4.23 g; 31.0 mmol), and CD_1–PEI (0.28 residue mM, 250 ml; 70 residue mmol). Lyophilization of the polymer produced yellow powders and the metal content of the polymer was 0.21% of the monomer residues.

$Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac was prepared through acetylation of $Zn^{II}_{0.2}$[CD_1–PEI–GO] according to the method described above for Ni^{II}_1[CD_1–PEI–GO]Ac.

Co^{II}_2[CD_1–PEI–BD] was prepared through the Co^{II}-template condensation of CD_1–PEI with glyoxal according to the method described above for Ni^{II}_1[CD_1–PEI–GO] by butanedione (1.44 g; 16.7 mmol), $CoCl_2 \cdot 6H_2O$ (7.94 g; 33.4 mmol), and CD_1–PEI (0.28 residue mM, 300 ml; 84 residue mmol). Lyophilization of the polymer produced dark-brown powders and the metal content of the polymer was 2.4% of the monomer residues. A previous study indicated that the macrocyclic Co^{II} center built on PEI is stable against oxidation under the conditions of the present investigation (*19*).

Co^{II}_2[CD_1–PEI–BD]Ac was prepared through acetylation of Co^{II}_2[CD_1–PEI–BD] according to the method described above for Ni^{II}_1[CD–PEI–GO]Ac.

Metal content of the PEI-based macrocyclic complexes was determined by ICP analysis.

Ester Substrates

4-Nitrophenyl(4-t-butylphenoxy)acetate (**1**). To a solution of *t*-butylphenol (3 g, 20 mmol) and NaOH (2.1 g; 53 mmol) in 200 ml 5% (v/v) ethanol–water, an aqueous solution of chloroacetic acid (2.0 g, 21 mmol) was added and the resulting solution was heated for 6 h on a steam bath. The resulting mixture was cooled to room temperature and acidified with 3 N HCl to obtain (4-*t*-butylphenoxy)acetic acid, which was recrystallized from ethanol, mp 81–84°C. ^{1}H NMR ($CDCl_3$); δ 1.29 (s, 9H), 4.65 (s, 2H), 6.79–7.38 (m, 4H), 8.65 (s, 1H). To a solution of (4-*t*-butylphenoxy)acetic acid (0.55 g, 2.6 mmol) in 50 ml tetrahydrofuran, *N*,*N*′-dicyclohexylcarbodiimide (0.65 g, 3.2 mmol) and 4-*N*,*N*-dimethylaminopyridine (0.1 g; catalytic amount) were added and the mixture was stirred for 30 min at room temperature. To the mixture, 4-nitrophenol (0.30 g, 2.6 mmol) was added and the mixture was stirred for 5 h at room temperature. The filtrate obtained by filtration was evaporated *in vacuo*, and the resulting residue (**1**) was recrystallized from

diethyl ether–hexane, mp 79–80°C. ^{1}H NMR ($CDCl_3$): δ 1.30 (s, 9H), 4.88 (s, 2H), 6.85–8.33 (m, 8H).

4-Nitrophenyl N-(4-t-butylbenzoyl)-4-aminobutyrate (**2**). The mixture of 4-*t*-butylbenzoic acid (1.0 g, 5.6 mmol) and 20 ml thionyl chloride was stirred for 6 h at 50°C. The oil (4-*t*-butylbenzoyl chloride) obtained after evaporation of excess thionyl chloride *in vacuo* was added to an aqueous solution (10 ml) of 4-aminobutyric acid (0.58 g, 5.6 mmol) and K_2CO_3 (1.9 g, 14 mmol) kept in an ice bath. After the mixture was stirred at room temperature overnight, it was acidified with 3 *N* HCl. The precipitate (*N*-(4-*t*-butylbenzoyl)-4-aminobutyric acid) was collected and washed with diethyl ether and recrystallized from ethyl acetate–hexane, mp 119–122°C. ^{1}H NMR (DMSO-d_6) δ: 1.28 (s, 9H), 1.52–1.92 (m, 2H), 2.16–2.36 (t, 2H), 3.14–3.28 (q, 2H), 7.35–7.64 (m, 4H), 8.26–8.30 (t, 1H), 11.98 (broad s, 1H). To a solution of *N*-(4-*t*-butylbenzoyl)-4-aminobutyric acid (0.15 g, 0.57 mmol) in 50 ml tetrahydrofuran, *N*,*N*′-dicyclohexylcarbodiimide (0.13 g, 0.63 mmol) and 4-*N*,*N*-dimethylaminopyridine (5 mg; catalytic amount) were added and the mixture was stirred for 30 min. After 4-nitrophenol (80 mg, 0.57 mmol) was added, the solution was further stirred for 5 h at room temperature. The filtrate obtained by filtration was evaporated *in vacuo*, and the resulting residue was recrystallized from ethyl acetate–hexane, mp 103–104°C. ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 9H), 1.91–2.25 (m, 2H), 2.65–2.82 (t, 2H), 3.49–3.73 (q, 2H), 6.34 (s, 1H), 7.16–8.30 (m, 8H).

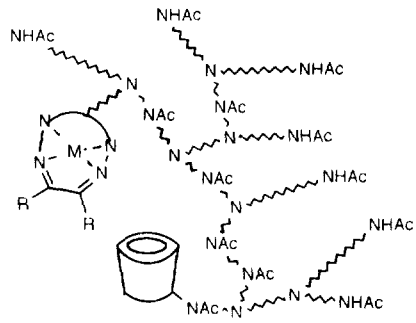
Kinetic Measurements

Reaction rates for the hydrolysis of **1** and **2** in the presence of the PEI derivatives were measured by monitoring the release of *p*-nitrophenol at 410 nm with a Beckman DU-64 uv/vis spectrophotometer. Temperature was controlled at 25 ± 0.1°C with a Haake E12 circulator. Reactions were carried out in the presence of 10% (v/v) acetonitrile which was used as the solvent for the stock solutions of the substrates. Water was distilled and deionized prior to use in kinetic measurements. Buffers (0.05 M) used were *N*-(2-hydroxyethyl)piperazinyl-*N*′-2-ethanesulfonate (pH 7 and 7.5) and tris(hydroxymethyl)aminomethane (pH 8 and 8.5).

RESULTS

As artificial metalloesterases containing CD cavities as binding sites, PEI derivatives such as Ni^{II}_1[CD_1–PEI–GO]Ac, Co^{II}_2[CD_1–PEI–BD]Ac, and $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac are prepared in the present study. As schematically illustrated in Scheme **3**, macrocyclic metal (M = Ni^{II}, Co^{II}, or Zn^{II}) complexes formed by the metal-template condensation (*15*) of the amino groups of PEI with glyoxal (R = H) or butanedione (R = CH_3) provide the metal centers of the artificial metalloesterases. The primary and secondary amino groups of the PEI derivatives are acetylated.

The pseudo-first-order rate constants k_0 measured for the hydrolysis of **1** in the presence of the PEI-based artificial metalloesterases at 25°C are summarized in



3 (M: Ni^{II}, Co^{II}, Zn^{II})
(R: H, CH_3)

SCHEME 3

Fig. 1. Rate data were measured at several concentrations of the catalysts (C_0)[3] under the conditions of $C_0 \gg S_0$. In the analysis of the kinetic data, C_0 (the initially added concentration of the catalyst) is expressed in terms of the concentration of CD residue of the catalyst.

Reactions catalyzed or promoted by PEI derivatives proceed through the formation of complex between the PEI derivative and the substrate (Eq. [1]; K_m is the dissociation constant for the CS complex) (*11–20*). The pseudo-first-order rate constant (k_0) for Eq. [1] is derived as Eq. [2] under the conditions of $C_0 \gg S_0$ (S_0, initially added concentration of the substrate).[4] When $K_m \gg C_0$, k_0 is proportional to C_0 as predicted by Eq. [2]. The bimolecular rate constant (k_{bi}) obtained as k_0/C_0 under the conditions of $K_m \gg C_0$, therefore, corresponds to k_{cat}/K_m.

$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} C + P_i \qquad [1]$$

$$k_0 = k_{cat}C_0/(K_m + C_0) \qquad [2]$$

For $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac and Co^{II}_2[CD_1–PEI–BD]Ac, saturation kinetic behavior is observed and the values of k_{cat} and K_m estimated on the basis of Eq. [2] are summarized in Table 1. For Ni^{II}_1[CD_1–PEI–GO]Ac, however, k_0 was proportional to C_0 up to C_0 = 0.02 M, indicating $K_m \gg 0.02$ M. From the proportionality constant, k_{cat}/K_m for Ni^{II}_1[CD_1–PEI–GO]Ac is estimated and listed in Table 1.

For the hydrolysis of **2**, kinetic behavior was examined for $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac at several pHs. The values of kinetic parameters thus obtained are summarized in Table 1 and the pH dependence of k_{cat} is illustrated in Fig. 2.

In contrast to the catalytic activity of Ni^{II}_1[CD_1–PEI–GO]Ac, Co^{II}_2[CD_1–PEI–BD]Ac, and $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac, the hydrolysis of **1** or **2** was not catalyzed by [CD_1–PEI]Ac, the PEI-derivative lacking the macrocyclic metal centers.

[3] For Co^{II}_2[CD_1–PEI–BD]Ac, C_0 was not raised above the range indicated in Fig. 1 since the absorbance of the polymer became too large for correct spectrophotometric measurement of the release of nitrophenol from **1**.

[4] Or, more precisely, under the conditions of $C_0 \approx [C] \gg [CS]$.

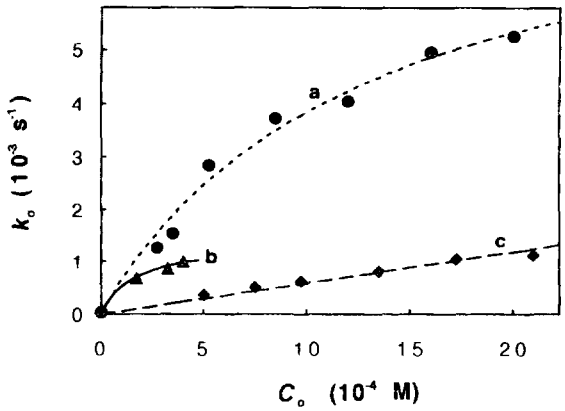


FIG. 1. The plot of k_0 against C_0 for the hydrolysis of **1** catalyzed by $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac (a), Co^{II}_2[CD_1–PEI–BD]Ac (b), and Ni^{II}_1[CD_1–PEI–GO]Ac (c) at 25°C and pH 7.5.

When the hydrolysis of **1** or **2** catalyzed by $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac was followed under the conditions of S_0 ($= 1 \times 10^{-3}$ M) $> C_0$ ($= 1 \times 10^{-4}$ M), no evidence was obtained for the inactivation of the catalyst although the hydrolysis was considerably faster than the spontaneous reaction. The catalytic sites, therefore, are regenerated during the hydrolysis reaction catalyzed by the metal-containing PEI derivative.

TABLE 1

Values of Kinetic Parameters for Ester Hydrolysis Catalyzed by the Poly(ethylenimine) Derivatives Containing both Macrocyclic Metal Centers and β-Cyclodextrin Cavities Measured at 25°C

Ester	Catalysts	pH	Parameter values
1	$Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac	7.50	$k_{cat} = (8.7 \pm 1.0) \times 10^{-3}$ s^{-1}
			$K_m = (1.3 \pm 0.3) \times 10^{-3}$ M
	Co^{II}_2[CD_1–PEI–BD]Ac	7.50	$k_{cat} = (1.4 \pm 0.2) \times 10^{-3}$ s^{-1}
			$K_m = (1.7 \pm 0.6) \times 10^{-4}$ M
	Ni^{II}_1[CD_1–PEI–GO]Ac	7.50	$K_m \gg 2 \times 10^{-2}$ M
			k_{bi} ($=k_{cat}/K_m$) $= 0.59 \pm 0.02$ s^{-1} M^{-1}
	[CD_1–PEI]Ac	7.50	No catalysis
2	$Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac	7.00	$k_{cat} = (2.3 \pm 0.2) \times 10^{-4}$ s^{-1}
			$K_m = (2.6 \pm 0.6) \times 10^{-4}$ M
		7.50	$k_{cat} = (5.9 \pm 0.5) \times 10^{-4}$ s^{-1}
			$K_m = (6.4 \pm 1.2) \times 10^{-4}$ M
		8.00	$k_{cat} = (6.5 \pm 0.7) \times 10^{-4}$ s^{-1}
			$K_m = (7.0 \pm 1.8) \times 10^{-4}$ M
		8.50	$k_{cat} = (7.6 \pm 0.3) \times 10^{-4}$ s^{-1}
			$K_m = (1.9 \pm 0.3) \times 10^{-4}$ M
	[CD_1–PEI]Ac	7.50	No catalysis

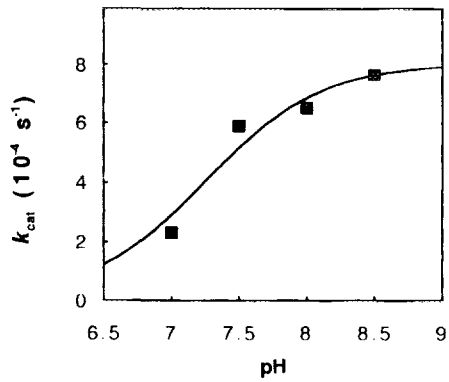


FIG. 2. The pH dependence of k_{cat} for the hydrolysis of **2** catalyzed by $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac at 25°C. The curve is drawn by assuming that the basic form of a monobasic acid with pK of 7.3 is the reactive species.

DISCUSSION

Structural features of the PEI-based artificial metalloesterases prepared in the present study are illustrated by **3**. For example, CD cavities, macrocyclic metal centers, tertiary amino groups, and acetylated primary and secondary amino groups are present on the PEI derivatives. In addition, the highly branched structure of PEI is also indicated in **3**. The exact molecular structure of the catalysts, however, is not shown in **3**. For example, the precise structure of macrocyclic complexes built on PEI and the relative positions of the CD moieties and the macrocyclic metal centers are not known.

As revealed by the previous study, CD_1–PEI recognizes esters containing *t*-butylphenyl moieties (*11*). Deacylation of nitrophenyl esters complexed with CD_1–PEI, however, occurs through the nucleophilic attack by the amino groups of the PEI backbone leading to inactivation of the polymer (*11*). In order to avoid complications in kinetic behavior arising from attack by the amino groups, the primary and secondary amino groups of the PEI derivatives examined in the present study are blocked by acetylation with excess acetic anhydride. Tertiary amines are not acetylated and a part of primary and secondary amines of the PEI backbone might be left unacetylated. Contribution of these unacetylated amines to the catalysis in the hydrolysis of **1** and **2** by the PEI-based artificial metalloesterases appears to be insignificant in view of the negligible activity of [CD_1–PEI]Ac (Table 1).

Lack of reactivity of [CD_1–PEI]Ac toward **1** and **2** (Table 1) indicates that the macrocyclic metal centers are essential to catalysis by $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac, Co^{II}_2[CD_1–PEI–BD]Ac, and Ni^{II}_1[CD_1–PEI–GO]Ac. The saturation kinetic behavior reveals complex formation between the ester substrates and the CD-containing PEI derivatives, in agreement with the results of previous studies on

binding of *t*-butylphenyl derivatives to CD derivatives (*8*, *11*). It is noteworthy that K_m values are widely different for $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac, Co^{II}_2[CD_1–PEI–BD]Ac, and Ni^{II}_1[CD_1–PEI–GO]Ac although the three polymers were prepared through metal-template condensation of the same CD_1–PEI with glyoxal.[5] Apparently, the environments around the CD cavities are considerably different for these three polymers, leading to the remarkable difference in the binding of **1**.

The metal centers in the PEI-based artificial metalloesterases may play specific catalytic roles (*13*, *14*) such as activation of electrophiles or provision of metal-bound hydroxide nucleophiles. It is also possible that the metal centers simply increase the effective local concentration of hydroxide ion on the polymer surface by electrostatic effects. The latter possibility, however, can be excluded on the basis of the greater reactivity of $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac compared with the Co^{II} or Ni^{II} analogs in spite of the much smaller content of Zn^{II} centers and, consequently, the much weaker electrostatic effects exerted by the Zn^{II} centers compared with the Co^{II} or Ni^{II} centers.

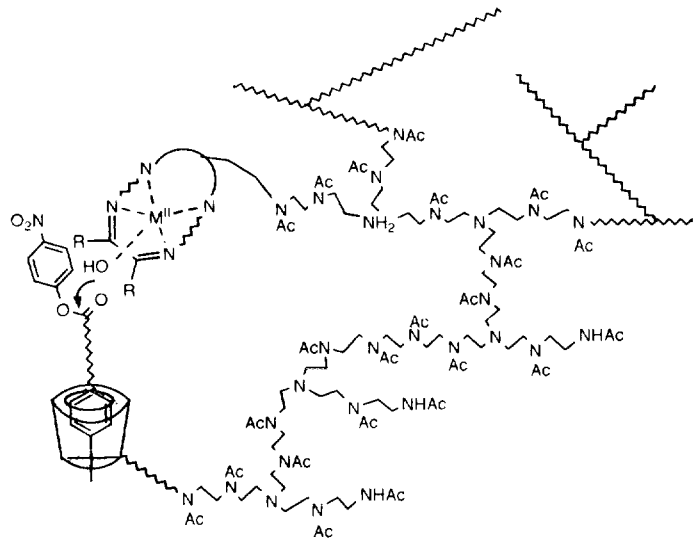
In order to obtain information useful for elucidation of the mechanism for the catalysis, the kinetic data were obtained at various pHs for the hydrolysis of **2** in the presence of $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac. As illustrated in Fig. 2, the pH dependence of k_{cat} reveals that the catalyst becomes active upon ionization of a functional group with pK_a of 7.3. This may be attributed to the ionization of the Zn^{II}-bound water molecules.

A mechanism consistent with the kinetic data is indicated by **4** (Scheme 4). The kinetic data, however, are also consistent with the mechanism involving complexation of the ester carbonyl group to the metal center and the subsequent attack of external hydroxide ion to the complexed ester. These two mechanisms would, however, produce almost identical tetrahedral intermediates.

In the ester hydrolysis by the PEI derivatives, the CD cavities provide binding sites for the substrates, recognizing the *t*-butylphenyl moieties. The macrocyclic metal centers of the PEI derivatives act as catalytic sites for the ester hydrolysis. In this regard, the PEI derivatives mimic metalloesterases. Since the macrocyclic metal centers and built randomly, only a small portion of the metal centers might be geometrically allowed to cooperate with the CD cavities. Even if the number of CD cavities cooperating with the metal centers is small, the PEI derivative can be regarded as an artificial metalloesterase, since enzymes also contain one or few active sites.

The content of Zn^{II} ion is 1/7 of that of CD in $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac. Only a small fraction of the CD cavities in $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac represent productive binding sites. In this regard, the scheme of Eq. [1] can be modified as Eq. [3]. Here, CS° represents the productive complex formed between the catalyst and the

[5] The contents of macrocyclic metal centers formed by the condensation of CD_1–PEI with glyoxal are considerably smaller than those (*19*) formed by condensation of PEI under identical conditions. Thus, the CD moieties in CD_1–PEI exert inhibitory effects on the metal-template condensation although the content (1.4% of the monomer residues) of CD in CD_1–PEI is much smaller than the total amount (25% of the monomer residue) of primary amines in PEI.



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SCHEME 4

substrate and $K°$ stands for [CS°]/[CS], the fraction of the productive complex. In addition, $k_{cat}°$ reflects the reactivity of productive complex CS°.

$$C + S \underset{K_m}{\rightleftharpoons} CS \overset{K°}{\rightleftharpoons} CS° \xrightarrow{k_{cat}°} C + P_i \quad [3]$$

The expression of k_0 derived from Eq. [3] under the condition of $C_0 \gg S_0$ is

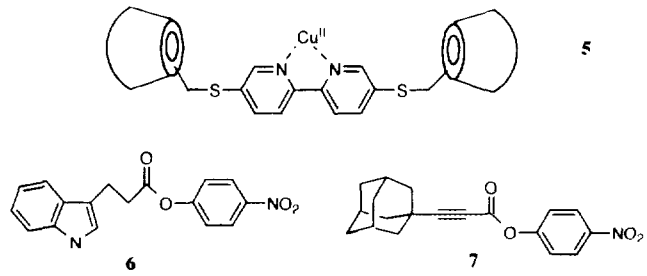
$$k_0 = k_{cat}^{app} C_0/(K_m^{app} + C_0) \quad [4]$$

$$k_{cat}^{app} = k_{cat}°K°/(1 + K°) \quad [5]$$

$$K_m^{app} = K_m/(1 + K°). \quad [6]$$

The values of k_{cat} and K_m listed in Table 1 correspond to those of k_{cat}^{app} and K_m^{app} when the scheme of Eq. [3] is operative. Since $K°$ is expected to be considerably smaller than 1, $k_{cat}°$ is to be much greater than the value of k_{cat} listed in Table 1. The nonproductive binding by the CD cavities of the PEI-based artificial metalloenzymes reduces the maximal rate ($V_{max} = k_{cat}^{app} C_0$) without affecting K_m^{app} appreciably.

Among the PEI-based artificial metalloesterases, the Zn^{II}-containing catalyst manifests the greatest k_{cat} value in spite of the smallest metal content. Considering the reduction in k_{cat}^{app} due to the nonproductive binding, the Zn^{II} centers appear to be much more effective in catalyzing the ester hydrolysis compared with Co^{II} or Ni^{II} centers. It is not clear, however, whether the greater reactivity of the Zn^{II} centers is due to the greater intrinsic catalytic capability of the metal ion or comes



SCHEMES 5, 6, AND 7

from better conformational relationship between the metal center and the bound ester.[6]

An example reported in the literature of artificial metalloenzyme containing CD cavities is CD dimer **5** (Schemes 5, 6, and 7), which manifested both complexation with substrate and catalytic turnover (*10*). Hydrolysis of esters **6** and **7** was catalyzed by **5** with the respective k_{cat} values of (0.5–1) $\times 10^{-3} s^{-1}$ and (0.7–1.2) $\times 10^{-2} s^{-1}$ at pH 7–8 and 37°C. Very large binding constants of **6** and **7** to **5** suggest accommodation of the esters by both of the two CD cavities of **5** and catalysis by the metal center in the hydrolysis of the esters bound to **5**. It is interesting to note that k_{cat} values for the hydrolysis of **1** or **2** catalyzed by $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac are not much different from those of **6** or **7** catalyzed by **5** when the difference in temperature is considered. Although the intrinsic reactivity of **6** and **7** toward deacylation (e.g., k_{OH}) is not known, the similar k_{cat} values measured for $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac and **5** suggest that the metal centers randomly built on $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac are as effective as the metal centers deliberately introduced between the two CD cavities of **5**.

In the design of effective artificial enzymes, both enhancement in k_{cat} (or k_{cat}^{app}) and reduction in K_m (or K_m^{app}) are to be achieved. Strategies to increase k_{cat}^{app} for the artificial enzymes built on PEI include introduction of reactive catalytic functional groups in close proximity to the bound substrate and cooperation among catalytic groups. In addition, it is also desirable to reduce the nonproductive binding, which may be accomplished by the introduction of catalytic groups in planned positions close to the binding sites. In this regard, our next step toward elaboration of the PEI-based artificial metalloenzymes involves the site-directed construction of metal centers in the vicinity of the CD cavities of CD-containing PEI.

[6] Rates of the spontaneous hydrolysis of **1** and **2** were measured at pH 9–11 and 25°C in the presence of 0.01–0.1 M borate, bicarbonate, or carbonate buffers (ionic strength = 0.5 M with NaCl, in the presence of 1.25% (v/v) acetonitrile), and the second-order rate constants (k_{OH}) for the attack of hydroxide ion at the esters were estimated as 73 ± 3 $M^{-1} s^{-1}$ for **1** and 13 ± 0.4 $M^{-1} s^{-1}$ for **2**. The k_{cat} measured for $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac at pH 7.50 is 15 times greater for **1** than for **2**, whereas the k_{OH} measured for the spontaneous alkaline hydrolysis is 6 times greater for **1** than for **2**. The difference in the relative reactivity of $Zn^{II}_{0.2}$[CD_1–PEI–GO]Ac and OH^- toward **1** and **2** is only 2.5, which is not large enough to suggest greater selectivity of the catalyst toward **1** than toward **2**.

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