

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. © 1994 Academic Press, Inc.

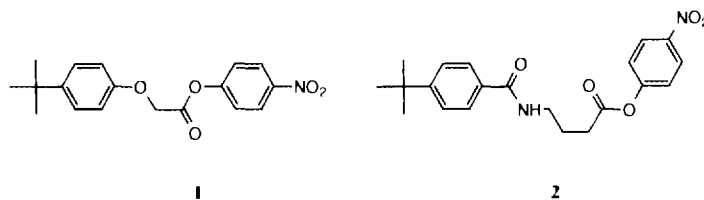
INTRODUCTION

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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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 CD₁-PEI in which the molar content of the CD moiety was 1.4% of the monomer residues of PEI.² [CD₁-PEI]Ac was prepared by acetylation of CD₁-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.

$\text{Ni}^{\text{II}}_1[\text{CD}_1\text{-PEI-GO}]$ was prepared through the Ni^{II} -template condensation of $\text{CD}_1\text{-PEI}$ with glyoxal according to the general procedure (17) described previously for $\text{Ni}^{\text{II}}_7[\text{PEI-GO}]$ by using glyoxal (40% solution, 1.30 g; 22.4 mmol), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (8.57 g; 36.1 mmol) and $\text{CD}_1\text{-PEI}$ (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.

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

$\text{Zn}^{\text{II}}_{0.2}[\text{CD}_1\text{-PEI-GO}]$ was prepared through the Zn^{II} -template condensation of $\text{CD}_1\text{-PEI}$ with glyoxal according to the method described above for $\text{Ni}^{\text{II}}_1[\text{CD}_1\text{-PEI-GO}]$ by using glyoxal (40% solution, 1.30 g; 22.4 mmol), ZnCl_2 (4.23 g; 31.0 mmol), and $\text{CD}_1\text{-PEI}$ (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.

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

$\text{Co}^{\text{II}}_2[\text{CD}_1\text{-PEI-BD}]$ was prepared through the Co^{II} -template condensation of $\text{CD}_1\text{-PEI}$ with glyoxal according to the method described above for $\text{Ni}^{\text{II}}_1[\text{CD}_1\text{-PEI-GO}]$ by butanedione (1.44 g; 16.7 mmol), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (7.94 g; 33.4 mmol), and $\text{CD}_1\text{-PEI}$ (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).

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

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. ^1H 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. ^1H 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. ^1H NMR ($\text{DMSO}-d_6$) 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. ^1H 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).

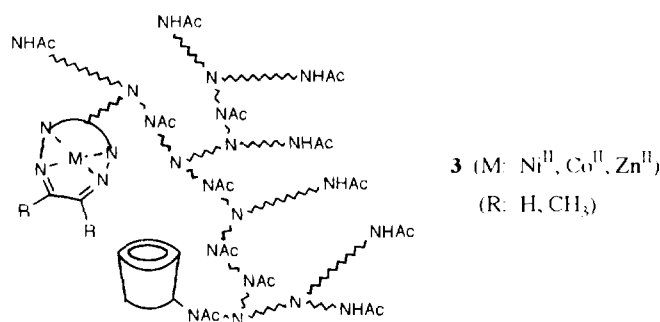
Kinetic Measurements

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 $25 \pm 0.1^\circ\text{C}$ 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 $\text{Ni}^{\text{II}}_1[\text{CD}_1\text{-PEI-GO}]\text{Ac}$, $\text{Co}^{\text{II}}_2[\text{CD}_1\text{-PEI-BD}]\text{Ac}$, and $\text{Zn}^{\text{II}}_{0.2}[\text{CD}_1\text{-PEI-GO}]\text{Ac}$ are prepared in the present study. As schematically illustrated in Scheme 3, macrocyclic metal ($\text{M} = \text{Ni}^{\text{II}}$, Co^{II} , or Zn^{II}) complexes formed by the metal-template condensation (15) of the amino groups of PEI with glyoxal ($\text{R} = \text{H}$) or butanedione ($\text{R} = \text{CH}_3$) provide the metal centers of the artificial metalloesterases. The primary and secondary amino groups of the PEI derivatives are acetylated.

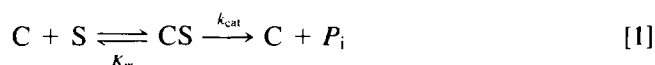
The pseudo-first-order rate constants k_0 measured for the hydrolysis of **1** in the presence of the PEI-based artificial metalloesterases at 25°C are summarized in



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 .



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

For Zn^{II}_{0.2}[CD₁–PEI–GO]Ac and Co^{II}₂[CD₁–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[CD₁–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[CD₁–PEI–GO]Ac is estimated and listed in Table 1.

For the hydrolysis of **2**, kinetic behavior was examined for Zn^{II}_{0.2}[CD₁–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[CD₁–PEI–GO]Ac, Co^{II}₂[CD₁–PEI–BD]Ac, and Zn^{II}_{0.2}[CD₁–PEI–GO]Ac, the hydrolysis of **1** or **2** was not catalyzed by [CD₁–PEI]Ac, the PEI-derivative lacking the macrocyclic metal centers.

³ For Co^{II}₂[CD₁–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 $C_0 \approx [C] \gg [CS]$.