BIOORGANIC CHEMISTRY **26,** 103–117 (1998)
ARTICLE NO. BH981090

Artificial Metalloesterases Constructed by Site-Directed Attachment of Oximinato Metal Centers to Poly(ethylenimine) Containing β-Cyclodextrin

Junghun Suh[1] and Won Jong Kwon

Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-742, Korea

Received February 11, 1998

In an effort to establish a methodology for construction of active sites of artificial enzymes, site-directed attachment of 2,6-diacetylpyridineketoxime (**III**) to poly(ethylenimine) (PEI) containing β-cyclodextrin (CD) was attempted. The site-directed functionalization exploited a *t*-butylphenyl ester (**I**) of a carboxylic acid containing a precursor of **III.** Anchoring of the *t*-butylphenyl group of **I** by the CD portion followed by transfer of **III** moiety to an amino group located in the vicinity of the CD moiety would lead to introduction of **III** in proximity to the CD moiety. By acylation in DMSO of CD-PEI with the phenyl ester (**II**), instead of the *t*-butylphenyl ester, **III** was introduced randomly to CD-PEI. In the presence of the Ni(II) or Zn(II) complex of the **III**-containing CD-PEI prepared by either the site-directed or the random functionalization method, ester hydrolysis of 4-(4′-acetoxy-phenylazo)-benzenesulfonate (**IV**) was considerably enhanced. Analysis of the kinetic data measured at various pHs revealed that k_{cat} for the PEI derivative prepared by site-directed modification was three to six times greater than that by random modification. The results were taken to indicate that **I** transferred **III** to the vicinity of the CD moiety of CD-PEI, but that orientation of **III** and the CD cavity in the resulting PEI derivative was not very productive for deacylation of **IV** complexed by the CD cavity.

INTRODUCTION

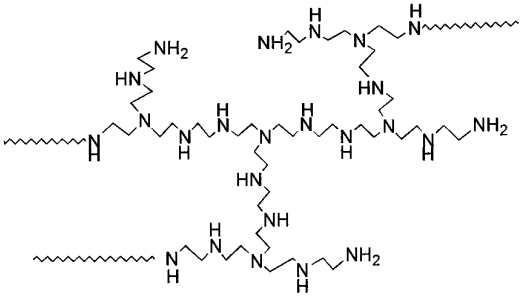
In devising artificial enzymes, it is necessary to incorporate catalytic elements exploited by enzymes in relatively small spaces on synthetic molecules. Furthermore, in order to achieve cooperative catalytic participation of several functional groups in the chemical transformation of the bound substrate, fine alignment of convergent catalytic groups is needed. Creation of such active sites on molecular skeletons provided by relatively small synthetic hosts would not be easy. For this reason, branched poly(ethylenimine) (PEI) has been exploited as a molecular backbone in an approach to the design of artificial enzymes (*1*).

PEI contains ethylamine as the repeating unit and, thus, is highly soluble in water. The molecular weight of the PEI used in the construction of artificial enzymes is ca. 60,000, corresponding to 1,400 monomer residues. Among the 1,400 nitrogens present in PEI, ca. 25, 50, and 25% are primary, secondary, and tertiary amines,

[1] To whom correspondence should be addressed.

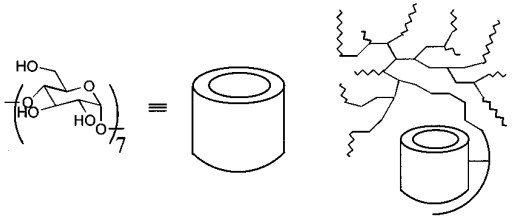
0045-2068/98 $25.00

respectively. The tertiary amines represent branching points on the polymer backbone, and PEI is highly branched. The amino nitrogens of PEI can be easily modified by alkylation, acylation, or imine-formation, allowing incorporation of various catalytic elements to the backbone.



PEI

One of the basic obstacles to overcome in the design of artificial enzymes by using synthetic polymers is the lack of specific binding sites on the macromolecular backbone. As for binding sites for certain hydrophobic moieties, β-cyclodextrin (CD) has been attached to PEI to obtain CD-PEI[2] (*2*). CD-PEI manifested high affinity toward *t*-butylphenyl compounds and accelerated deacylation of esters containing *t*-butylphenyl moieties. In a subsequent study, macrocyclic metal complexes have been randomly built on the PEI backbone of CD-PEI (*3*), which acted as effective catalytic groups for ester hydrolysis.



CD **CD-PEI**

As a further step toward design of effective artificial enzymes based on CD-PEI, it is needed to develop methodology for introduction of additional catalytic elements in the vicinity of the CD cavity by site-directed functionalization (*4*). Functionalization of CD-PEI can be also regarded as preparation of new derivatives (*5*) of CD.

In the present study, site-directed functionalization of CD-PEI was undertaken for introduction of oximinato metal centers. Attachment of oximinato metal centers in proximity to the CD cavity could lead to cooperation by metal centers and the oxime group as catalytic groups as well as the CD cavity as the binding site. Previous

[2] The abbreviated names of the PEI derivatives indicate nature of the pendants (CD, Ac, DAP, DAPOx, or M(II)) attached to PEI and whether DAP is introduced by site-directed or random (SD or Ran, respectively) functionalization. For example, [Zn(II)DAPOx-CD]^{SD}AcPEI stands for the PEI derivative containing CD and acetyl groups as well as the Zn(II) complex of DAPOx introduced by site-directed functionalization.

studies (*6*) showed that attack of 2-pyridyl oxime groups at esters and breakdown of the resulting carboxy oxime esters are catalyzed by the metal ion bound to the oxime nitrogen. In this article, introduction of oximinato metal complexes to CD-PEI by site-directed or random functionalization is described together with kinetic data for ester deacylation by the resulting artificial metalloesterases.

EXPERIMENTAL PROCEDURES

Materials

4-t-Butylphenyl 4-(6-acetyl-pyridin-2-yl)-4-oxobutyrate (**I**). A benzene solution (100 ml) of 2,6-diacetylpyridine (16 g, 0.10 mol), ethyleneglycol (6.2 g, 0.10 mol), and *p*-toluenesulfonic acid (0.20 g, 0.20 mol) was refluxed for 1 h. The solvent was evaporated under a reduced pressure and the resulting residue was purified by column chromatography (SiO_2, 6 : 1 hexane-ethyl acetate) to obtain 1-[6-(2-methyl-[1,3]dioxola-2-*yl*)-pyridin-2-*yl*]-ethanone (**Ia**) as a pale yellow oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.82 (s, 3H), 2.76 (s, 3H), 3.99 (m, 2H), 4.15 (m, 2H), 7.75 (dd, 1H), 7.85 (t, 1H), 7.89 (dd, 1H). To a solution of **Ia** (1.0 g, 4.8 mmol) in tetrahydrofuran (THF) (20 ml) was added sodium bis(trimethylsilyl)amide (5.8 ml of 1.0 M solution in THF, 5.8 mmol) and the mixture was stirred for 30 min at −78°C. After addition of ethyl bromoacetate (0.53 ml, 4.8 mmol), the reaction mixture was stirred for 1 h at −20°C. After quenching the reaction by adding aqueous 5% $NaHCO_3$ (100 ml), the reaction mixture was extracted with CH_2Cl_2 and the residue obtained by evaporation of the organic layer under a reduced pressure was purified by column chromatography (SiO_2, 4 : 1 hexane-ethyl acetate) to obtain ethyl 4-[6-(2-methyl-[1,3]dioxolan-2-*yl*)-pyridin-2-*yl*]-4-oxobutyrate (**Ib**) as a pale yellow oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.82 (s, 3H) 2.75 (t, 2H), 3.62 (t, 2H), 3.97 (m, 2H), 4.14 (m, 2H), 7.75 (dd, 1H), 7.85 (t, 1H), 7.98 (dd, 1H). Ester **Ib** was hydrolyzed by refluxing in 1 : 1 THF-3 N HCl (100 ml) for 3 h. The product was extracted with CH_2Cl_2 and the residue, 4-(6-acetyl-pyridin-2-*yl*)-4-oxobutyric acid (**Ic**), obtained by evaporation of the solvent was used in the next step without further purification: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.80 (s, 3H), 2.86 (t, 2H), 3.63 (t, 2H), 8.00 (t, 1H), 8.23 (m, 2H). To a solution of **Ic** (1.0 g, 2.8 mmol) and *t*-butylphenol (0.87 g, 5.8 mmol) in CH_2Cl_2 were added *N*,*N*′-dicyclohexylcarbodiimide (1.2 g, 5.8 mmol) and 4-*N*,*N*-dimethylaminopyridine (0.1 g) and the mixture was stirred for 1 h at 0°C. After evaporation of the solvent, the residue was dissolved in 1 : 1 ethyl acetate-hexane (100 ml). After removal of undissolved solids, the solution was concentrated and purified by column chromatography (SiO_2, 6 : 1 hexane-ethyl acetate) and recrystallization from hexane-ether to obtain **I** as white crystals, mp 71–73°C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30 (s, 9H), 2.80 (s, 3H), 3.06 (t, 2H), 3.76 (t, 2H), 7.04 (m, 2H), 7.48 (m, 2H), 8.00 (t, 1H), 8.23 (m, 2H). FAB-MS 354 (MH+). Anal. Calcd. for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96. Found: C, 71.09; H, 6.72; N, 3.86.

Phenyl 4-(6-acetyl-pyridin-2-yl)-4-oxobutyrate (**II**). This compound was prepared by the method used for preparation of **I** by using phenol instead of 4-*t*-butylphenol, mp 67–68°C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.80 (s, 3H), 3.06 (t, 2H), 3.76 (t, 2H),

7.04 (m, 2H), 7.32 (m, 1H), 7.48 (m, 2H), 8.00 (t, 1H), 8.23 (m, 2H). FAB-MS 298 (MH+). Anal. Calcd. for $C_{17}H_{25}NO_4$: C, 68.69; H, 5.09; N 4.71. Found: C, 68.42; H, 4.92; N, 4.63.

CD-PEI. By the procedure reported previously (*2*), CD was attached to PEI and the content of CD in the purified CD-PEI was estimated as 1.4 residue mol %.

[*DAP-CD*]^{SD}*PEI.* To a solution of 0.162 residue M CD-PEI in 650 ml water and 100 ml DMSO, **I** (0.598 g, 1.69 mmol) dissolved in 50 ml DMSO was added dropwise over a period of 1.5 h at 40°C, and the solution was stirred at 40°C for 3 days. The resulting PEI derivative, [DAP-CD]^{SD}PEI, was purified by dialysis against 30% (v/v) ethanol-water (thrice), saturated NaCl solutions (twice), and water (thrice).

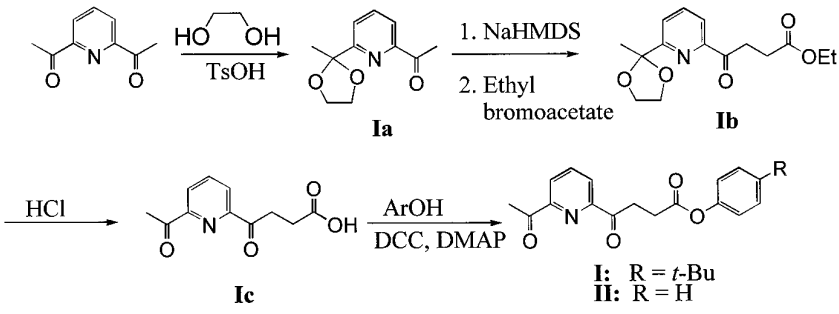
[*DAP-CD*]^{Ran}*PEI.* An aqueous solution of 0.242 residue M CD-PEI (100 ml) was evaporated under a reduced pressure below 40°C and the resulting dry polymer was dissolved in 200 ml DMSO. After addition of **II** (0.101 g, 0.339 mmol), the reaction mixture was stirred at 40°C for 3 days. The resulting PEI derivative, [DAP-CD]^{Ran}PEI, was purified by dialysis as described above for [DAP-CD]^{SD}PEI.

[*DAPOx-CD*]^{SD}*PEI and* [*DAPOx-CD*]^{Ran}*PEI.* To a solution of [DAP-CD]^{SD}PEI or [DAP-CD]^{Ran}PEI (0.242 residue M, 250 ml, pH 7.0), hydroxylamine hydrochloride (14 g, 200 mmol) dissolved in 10 ml water was added and the resulting solution was stirred for 6 days at 40°C. The resulting PEI derivatives, [DAPOx-CD]^{SD}PEI and [DAPOx-CD]^{Ran}PEI, were purified by dialysis against saturated NaCl solutions (thrice) followed by water (four times).

[*DAPOx-CD*]^{SD}*AcPEI and* [*DAPOx-CD*]^{Ran}*AcPEI.* Acetylation of [DAPOx-CD]^{SD}PEI or [DAPOx-CD]^{Ran}PEI was carried out with acetic anhydride as reported previously (*3*, *4*). The mixture was stirred at pH 12 for 12 h to hydrolyze acetyl oxime that might have been formed during the acetylation stage. After neutralization to pH 7, the resulting PEI derivatives, [DAPOx-CD]^{SD}PEI and [DAPOx-CD]^{Ran}PEI, were purified by dialysis against saturated NaCl solutions (thrice) followed by water (four times).

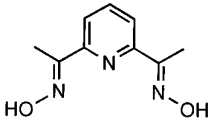
2,6-Diacetylpyridine dioxime (**III**). To a solution of 2,6-diacetylpyridine (3.3 g, 20 mmol) in ethanol (50 ml), an aqueous solution (20 ml) of hydroxylamine hydrochloride (1.5 g, 22 mmol) was added and the mixture was refluxed for 4 h. The residue obtained by evaporation of the solvent under a reduced pressure was recrystallized from ethyl acetate-hexane to obtain **III,** mp 252–254°C (dec). ^{1}H NMR (80 MHz, DMSO-d_6) δ 2.27 (s, 6H), 7.80 (t, 1H), 8.02 (d, 2H). FAB-MS 194(MH^+). Anal. Calcd. for $C_9H_{11}N_3O_2$: C, 55.95; H, 5.74; N, 21.75. Found: C, 55.84; H, 5.68; N, 21.82.

4-(4′-Acetoxy-phenylazo)-benzenesulfonic acid (**IV**). An aqueous solution (10 ml) of sodium nitrite (0.75 g, 11 mmol) was poured into a concentrated HCl solution (150 ml) containing sulfanilic acid (1.7 g, 10 mmol) at 0°C. The reaction mixture was stirred for 30 min in an ice bath and then added dropwise into a solution of phenol (1.0 g, 11 mmol) and sodium carbonate (100 g, 1.0 mol) in 4:1 water-methanol (250 ml) at 0°C. The reaction mixture was stirred for 1 h and then acidified with 1 *N* HCl (100 ml). The precipitates thus obtained were purified by recrystallization from ethanol to obtain 4-(4′-hydroxy-phenylazo)-benzenesulfonic acid (**IVa**), mp 282–284°C (dec). That the azo coupling reaction took place at the para position of phenol was confirmed by ^{13}C NMR data. ^{1}H NMR (80 MHz,

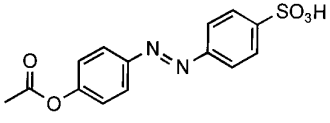


SCHEME 1

DMSO-d_6) δ 6.98 (d, 2H), 7.80 (m, 6H), 10.32 (s, 1H). ^{13}C NMR (80 MHz, DMSO-d_6) δ 116.01, 121.63, 126.69, 143.24, 145.27, 149.24, 152.03, 161.12. Anal. Calcd. for $C_{12}H_{10}N_2O_4S$: C, 51.79; H, 3.62; N, 10.07; S, 11.52. Found: C, 51.53; H, 3.77; N, 9.97; S, 11.43. The solution of **IVa** (2.8 g, 10 mmol) in 1 : 6 DMF-acetic anhydride (350 ml) was stirred for 4 h at 50°C and then cooled in an ice bath. The precipitates were purified by recrystallization from ethanol to obtain **IV,** mp 162–163°C (dec). ^{1}H NMR (80 MHz, DMSO-d_6) δ 2.31 (s, 3H), 6.94 (d, 2H), 7.80 (m, 6H). FAB-MS 321(MH^+). Anal. Calcd. for $C_{14}H_{12}N_2O_5S$: C, 52.49; H, 3.78; N, 8.75; S, 10.10. Found: C, 52.31; H, 3.84; N, 8.63; S; 9.89.



III



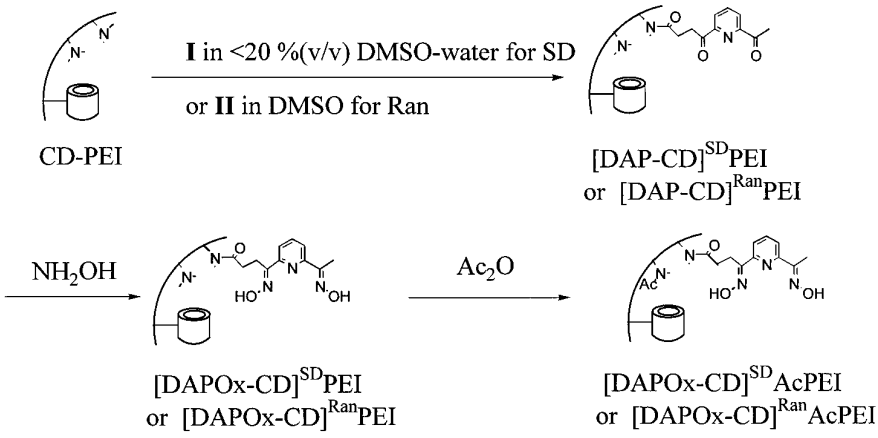
IV

Measurements

Kinetic studies for deacylation of **IV** were carried out spectrophotometrically with a Beckman DU 650 UV-Vis spectrophotometer. pH was adjusted with a Dongwoo Medical System pH/ion meter DP 880. Buffers (0.05 M) used were sodium acetate (pH 4.8), 2-[*N*-morpholino]ethanesulfonate (pH 5.8–6.5) and *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (pH 7–8).

RESULTS

The *t*-butyl phenyl ester (**I**) and the phenyl ester (**II**) of 4-(6-acetyl-pyridin-2-*yl*)-4-oxobutyric acid were prepared according to the synthetic route summarized in Scheme 1. As summarized in Scheme 2, CD-PEI was coupled with **I** in 13–19% (v/v) DMSO-water to obtain [DAP-CD]^{SD}PEI. By treatment with NH_2OH, [DAP-CD]^{SD}PEI was converted into [DAPOx-CD]^{SD}PEI, the corresponding oxime. Acetylation of primary and secondary amino groups of the PEI backbone with acetic



SD: site-directed functionalization
Ran: random functionalization

SCHEME 2

anhydride transformed [DAPOx-CD]^{SD}PEI into [DAPOx-CD]^{SD}AcPEI. By coupling CD-PEI with **II** in DMSO, [DAP-CD]^{Ran}PEI was obtained, which was converted into [DAPOx-CD]^{Ran}PEI and [DAPOx-CD]^{Ran}AcPEI by further treatment with NH_2OH followed by acetic anhydride. It is known that treatment of PEI derivatives with acetic anhydride effectively acetylates primary and secondary amino groups of the PEI backbone (*3*, *4*).

The content of CD in CD-PEI was estimated as 1.4 residue mol% and, therefore, each CD-PEI molecule contained 20 CD moieties on the average. In the preparation of [DAP-CD]^{SD}PEI or [DAP-CD]^{Ran}PEI, the molar amount of **I** or **II** used was equivalent to that of the CD moieties. If the acylation of CD-PEI with **I** or **II** was quantitative, the content of the pyridyl moieties in the PEI derivatives subsequently prepared would be also 1.4 residue mol%. Elemental analysis of the PEI derivatives did not give reliable data for estimation of the content of the pyridyl moieties. Instead, the content of the oxime was estimated by comparing absorbance (305 nm) of [DAPOx-CD]^{SD}AcPEI or [DAPOx-CD]^{Ran}AcPEI with that of **III** in 3 M NaOH solutions. The content of **III** moiety thus estimated was 0.88 residue mol% for [DAPOx-CD]^{SD}AcPEI and 1.2 residue mol% for [DAPOx-CD]^{Ran}AcPEI. The number of **III** moiety for each CD moiety is 0.60 and 0.82, respectively, for [DAPOx-CD]^{SD}AcPEI and [DAPOx-CD]^{Ran}AcPEI.

Anionic ester **IV** was used as the substrate instead of neutral esters. This is because of the much greater solubility of the ionic ester that allows kinetic studies under the conditions of S_0 (initially added substrate concentration) $\gg C_0$ (initially added catalyst concentration). With neutral substrates, a large amount of an organic cosolvent is needed to raise the substrate concentration to a sufficiently high level

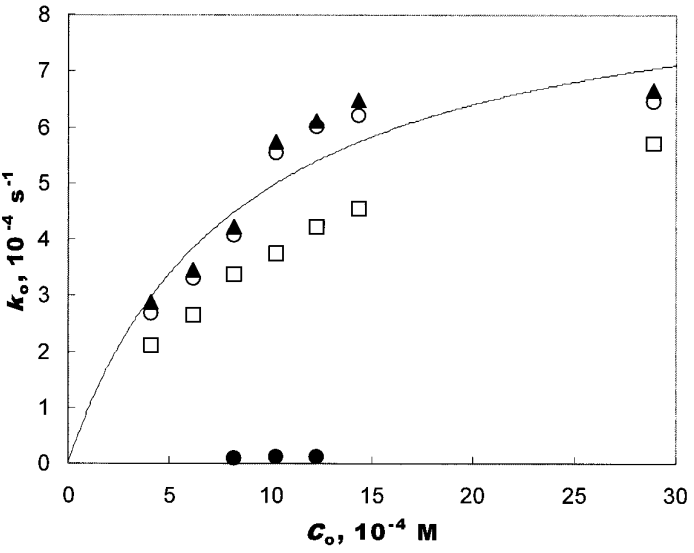


FIG. 1. Plot of k_0 against C_0 measured for the deacylation of **IV** in the presence of Ni(II) ion and [DAPOx-CD]^{SD}AcPEI at pH 5.88 and 25°C under the conditions of $C_0 \gg S_0$. The amount of Ni(II) ion added is 1.2 (▲), 1.0 (○), 0.8 (□), or 0 (●) equivalent of the oxime moiety of the PEI derivative. The curve is obtained by analysis with Eq. [2].

and the presence of such a cosolvent would interfere with the recognition of the hydrophobic substrate by the CD moiety of the catalyst.

Rates for deacylation of **IV** was studied in the presence of [DAPOx-CD]^{SD}AcPEI and [DAPOx-CD]^{Ran}AcPEI. When Ni(II) or Zn(II) was added to the reaction mixture, the rate was enhanced greatly. In Fig. 1, a sample of kinetic data obtained for deacylation of **IV** by the PEI derivatives in the presence of the bivalent metal ion added in various ratios relative to the oxime moieties is illustrated. When neither Ni(II) ion nor Zn(II) ion was added, the deacylation rate was much slower than the rate measured in the presence of the metal ions. This excludes the possibility of nucleophilic attack by the hydroxyl groups of the CD cavities or the amino groups of the PEI backbone. When tested at various pHs, the rate data indicated that addition of Ni(II) or Zn(II) ion in a 1 : 1 molar ratio to the oxime moieties of the PEI derivatives was sufficient to achieve maximal rates.

Kinetic data obtained with reactions catalyzed by PEI derivatives conform to the Michaelis–Menten scheme (Eq. [1]) (*1–4*). Under the conditions of $C_0 \gg S_0$,[3] Eq. [2] is derived from Eq. [1] as the expression of the pseudo-first-order rate constant (k_0). From dependence of k_0 on C_0 (expressed in terms of the concentration of the CD moieties) measured under the conditions of $C_0 \gg S_0$, values of k_{cat} and K_m were estimated. Values of the kinetic parameters estimated at various pHs for the Ni(II) or Zn(II) complexes of [DAPOx-CD]^{SD}AcPEI and [DAPOx-CD]^{Ran}AcPEI

[3] More accurately, $C_0 \sim [C] \gg [CS]$.