



Pergamon

Bioorganic &amp; Medicinal Chemistry Letters 11 (2001) 2401–2404

BIOORGANIC &  
MEDICINAL  
CHEMISTRY  
LETTERS

# Hydrolysis of Plasmid DNA Catalyzed by Co(III) Complex of Cyclen Attached to Polystyrene

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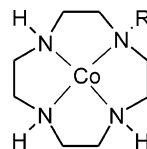
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Received 11 May 2001; accepted 26 June 2001

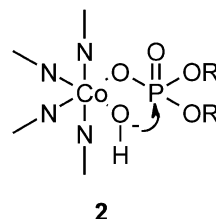
**Abstract**—Reactivity of the Co(III) complex of cyclen (CoCyc) in the hydrolytic cleavage of supercoiled pUC18 DNA leading to the formation of the corresponding open circular form was enhanced by >200 times upon attachment of CoCyc to cross-linked polystyrenes. Thus, half-lives as short as 40 min were achieved by the resin-based CoCyc in cleavage of the supercoiled DNA at 4 °C. © 2001 Elsevier Science Ltd. All rights reserved.

Phosphodiester linkages of DNA are very stable to hydrolysis. The half-life for spontaneous hydrolysis of the phosphodiester has been estimated as about 10<sup>11</sup> years at pH 7 and 25 °C.<sup>1</sup> To design artificial restriction enzymes, it is essential to secure synthetic catalytic centers effectively hydrolyzing the phosphodiester bonds. Most of known synthetic catalysts for DNA hydrolysis are metal complexes.<sup>2–14</sup>

The Co(III) complex of cyclen (CoCyc: **1a**) is one of the most effective synthetic catalysts discovered so far for DNA hydrolysis. It has been estimated that >10<sup>7</sup>-fold rate increase was achieved with CoCyc in hydrolysis of phosphodiester linkages leading to the conversion of a supercoiled DNA to the corresponding open circular form.<sup>9</sup> Hydrolytic nature of the DNA cleavage by the Co(III) complexes of polyamines including cyclen was evidenced by chemical or enzymatic religation<sup>10</sup> of the cleavage products as well as by the detection<sup>6</sup> of 3'-OH and 5'-OH termini of the cleavage products. A mechanism (**2**) has been proposed<sup>6</sup> for the catalytic action of the Co(III) complexes: the Co(III) center binds the phosphate anion of the phosphodiester linkage and the adjacent Co(III)-bound hydroxide ion makes intramolecular attack at the phosphorus atom of the bound substrate.



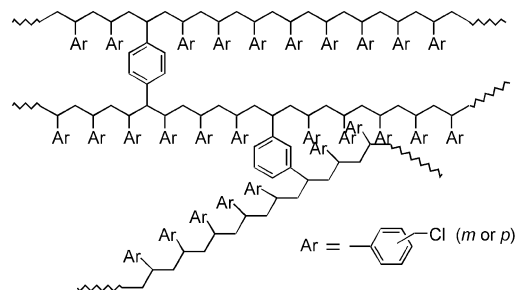
- 1a:** R = H  
**1b:** R = (CH<sub>2</sub>)<sub>n</sub>N<sup>+</sup>Me<sub>3</sub> (n = 3–6)  
**1c:** R = [(CH<sub>2</sub>)<sub>6</sub>N<sup>+</sup>Me<sub>2</sub>]<sub>n</sub>Me (n = 2, 3)  
**1d:** R = [(CH<sub>2</sub>)<sub>6</sub>N<sup>+</sup>Me<sub>2</sub>]<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>[(cyclen)Co]



In attempts to improve the catalytic activity of CoCyc, various derivatives of CoCyc (**1b–d**) were prepared.<sup>9</sup> The cationic substituents introduced in **1b–d** facilitated complexation between the catalyst and the DNA substrate, but the intrinsic reactivity of the Co center was little affected by the substituents.

In a previous study,<sup>15</sup> we observed that the protein-cleaving activity of the Cu(II) complex of Cyc was enhanced remarkably upon attachment to a derivative of poly(chloromethylstyrene-co-divinylbenzene) (PCD). PCD, an insoluble resin, is a cross-linked polystyrene in which the styrene monomer contains chloromethyl group. In the present study, we tested whether the activity of the Co(III) complex of Cyc (CoCyc) in phosphodiester hydrolysis is also enhanced greatly upon attachment to PCD derivatives.

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### PCD

PCD was synthesized by suspension copolymerization of chloromethylstyrene and divinylbenzene (2 mol% relative to chloromethylstyrene) according to a procedure reported in the literature<sup>16</sup> as described<sup>15</sup> previously. Synthetic route to one of the catalysts is summarized in Scheme 1. Substitution of chloromethyl groups of PCD with aminobutylguanidinium chloride, cyclen, or methoxide was carried out as reported previously.<sup>15</sup> Conversion of [Cyc][Gua]<sup>MeO</sup>PCD to [CoCyc][Gua]<sup>MeO</sup>PCD was carried out by following the procedure reported in the literature.<sup>9</sup> The content of CoCyc was estimated through measuring the amount of Co(III) ion released from the resin upon treatment with concentrated nitric acid by inductively coupled plasma-atomic emission spectroscopy. The content of guanidinium was measured by the binding experiment<sup>15</sup> with *p*-nitrobenzoate. The contents of CoCyc unit and guanidinium ion in [CoCyc][Gua]<sup>MeO</sup>PCD were 1.0 residue mol% (0.066 meq/g resin) and 0.81 residue mol% (0.052 meq/g resin), respectively. The residual chloromethyl groups of the resin were converted to methoxymethyl groups. Also synthesized was [CoCyc]<sup>MeO</sup>PCD, which lacks the guanidinium groups. The content of CoCyc in [CoCyc]<sup>MeO</sup>PCD was 0.95 residue mol% (0.061 meq/g resin). Dry beads of the PCD-based resins were swollen in water at 80 °C for 1 h, prior to use in kinetic studies. CoCyc was synthesized as reported.<sup>9</sup>

Plasmid pUC18 DNA (2686 base pairs) was used as the supercoiled DNA substrate. The plasmid DNA was prepared and isolated according to standard protocols.<sup>17</sup> The *Escherichia coli* cell line used for infection was XL1-Blue. The purity of pUC18 DNA was confirmed via both agarose gel electrophoresis and UV spectroscopy by determining the ratio of absorbance at

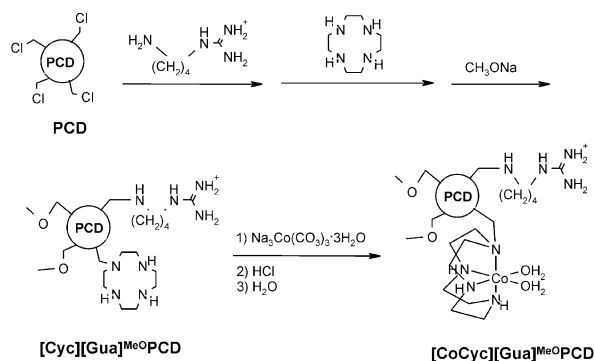
260 nm to the absorbance at 280 nm. The concentration of DNA was determined from the absorbance at 260 nm ( $A_{260} = 1.0$  for 50 µg/mL).

A solution (0.3 mL) containing the supercoiled DNA (39 nM) was incubated in a 1.5 mL Eppendorf tube with the PCD-based catalyst at 4 °C. The disappearance of the supercoiled DNA during incubation with the catalyst was monitored by agarose gel electrophoresis<sup>17</sup> with ethidium bromide staining, as exemplified in Figure 1. In this figure, disappearance of the supercoiled form is accompanied by appearance of the open circular form.<sup>18</sup> One nick in the supercoiled form brought by hydrolysis of a phosphodiester linkage causes the DNA to unwind into the open circular form<sup>19</sup> and the compact supercoiled form migrates through the gel pores more rapidly than the open circular form.

The pseudo-first-order rate constant ( $k_o$ ) for the cleavage of the supercoiled DNA was estimated by measuring the density of its electrophoretic bands.<sup>20</sup> For the PCD-based catalysts, reaction rate increased as the stirring speed was raised, reaching maximum values at  $\geq 800$  rpm. Thus, kinetic measurement was performed with the mixture stirred with a tiny magnetic bar at the stirring speed of 1000 rpm.

Kinetic data were collected at 4 °C under the conditions of  $C_o$  (concentration of CoCyc moiety obtainable when the PCD derivatives are assumed to be dissolved)  $> S_o$  (the initially added concentration of DNA). The dependence of  $k_o$  on  $C_o$  was measured as exemplified by the data summarized in Figure 2 and the pH dependence of  $k_2$  (proportionality constant for the plot of  $k_o$  against  $C_o$ ) is illustrated in Figure 3.

The reactivity [ $k_o = (4.2 \pm 0.8) \times 10^{-6} \text{ s}^{-1}$  for 1 mM  $C_o$  at 37 °C and pH 7.5] of CoCyc toward the supercoiled pUC18 measured in this study is comparable to the reported value ( $k_o = 1 \times 10^{-5} \text{ s}^{-1}$  for 1 mM  $C_o$  at 37 °C and pH 7.6)<sup>6</sup> for that toward supercoiled pUC9. When the supercoiled DNA was incubated with the Cyc-containing resins without Co(III) ion, cleavage of the DNA was negligible under the conditions of the kinetic measurements.



Scheme 1. Synthetic route to [CoCyc][Gua]<sup>MeO</sup>PCD.

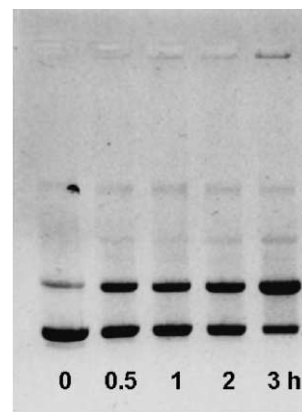
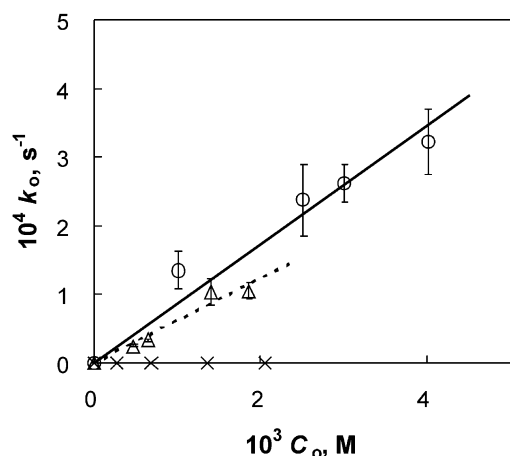
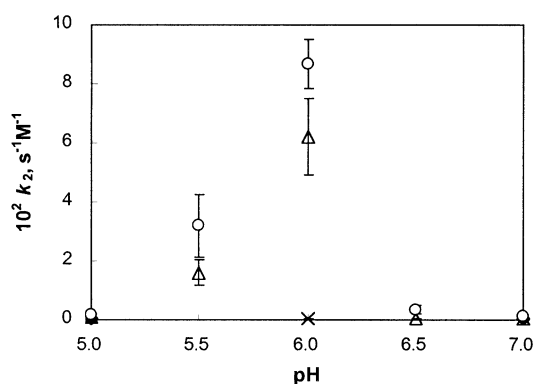


Figure 1. A typical negative image of electrophoretic separation showing conversion of the supercoiled form (lower bands) of plasmid pUC18 DNA into the open circular form (upper bands) by the CoCyc-based catalysts.



**Figure 2.** Plot of  $k_o$  against  $C_o$  for cleavage of the supercoiled pUC18 DNA by [CoCyc][Gua]<sup>Me</sup>OPCD (○), [CoCyc]<sup>Me</sup>OPCD (Δ), or CoCyc (×) at pH 6.0 and 4 °C. For CoCyc (up to 2 mM),  $k_o$  was smaller than  $0.8 \times 10^{-6} s^{-1}$  (half-life: > 10 days).



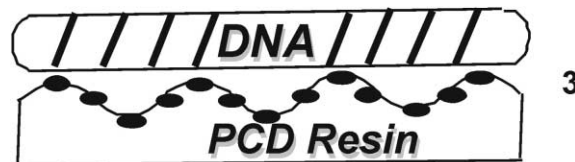
**Figure 3.** pH profile of  $k_2$  for cleavage of the supercoiled pUC18 DNA by [CoCyc][Gua]<sup>Me</sup>OPCD (○), [CoCyc]<sup>Me</sup>OPCD (Δ), or CoCyc (×) at 4 °C. For CoCyc,  $k_2$  was less than  $4 \times 10^{-4} s^{-1} M^{-1}$  at pH 5–7.

The rate for cleavage of the supercoiled DNA by [CoCyc][Gua]<sup>Me</sup>OPCD ( $C_o = 1$  mM) at pH 7.0 and 25 °C was not affected appreciably by additives such as catalase (240 μg/mL), superoxide dismutase (60 μg/mL), dimethyl sulfoxide (1.0 M), ethanol (2.0 M), and H<sub>2</sub>O<sub>2</sub> (0.6 M). As mentioned above, it is well established that the Co(III) complexes of polyamines including cyclen cleave DNA by hydrolysis. That the DNA cleavage observed in this study is also due to hydrolytic cleavage is supported by lack of appreciable effects on the reaction rates by catalase, superoxide dismutase, dimethyl sulfoxide, or ethanol. These inhibit the oxidative cleavage of DNA by diffusable free radicals, hydrogen peroxide, or superoxide.<sup>9,19,21</sup>

The highest value of  $k_o$  indicated in Figure 2 corresponds to the half-life of 40 min. Comparison of the rate constants measured for CoCyc and for the PCD-based CoCyc at the same  $C_o$  concentration reveals that the reactivity of CoCyc is enhanced upon attachment to PCD by more than 200 times toward the supercoiled DNA ([CoCyc][Gua]<sup>Me</sup>OPCD at pH 6.0). Considering that only the CoCyc moieties exposed on the surface of PCD can attack the DNA, the degree of activation should be much greater than 200-fold.<sup>22</sup> The facile hydrolytic cleavage of the supercoiled DNA by the

PCD-based CoCyc is, therefore, due to the activation of CoCyc upon attachment to PCD.<sup>23,24</sup>

When the DNA is complexed by the resin, several CoCyc moieties would be contained in the region covered by the DNA. When estimated by the method employed previously,<sup>15</sup> one CoCyc unit is present in the area of about  $2 \times 10^3 \text{ Å}^2$  on the surface of [CoCyc][Gua]<sup>Me</sup>OPCD. The area on the resin surface covered by the DNA with 2686 base pairs may be estimated as  $2 \times 10^5 \text{ Å}^2$  based on the dimensions of B-DNA (24 Å thickness of double helix and 3.4 Å length for each base pair). Thus, about 100 units of CoCyc are present on the resin surface covered by the DNA. The tubular DNA can occupy only wide open space on the resin whereas the resin surface is very rugged. Thus, only a small portion of the 100 CoCyc units can interact with the phosphate linkages, as illustrated by 3.<sup>25</sup> In 3, the stripes and the dark ellipses represent the phosphate linkages and the CoCyc moieties, respectively. Availability of several catalytic units for each complexed DNA is not, therefore, sufficient to account for the high reactivity of the Co(III)-containing resins.



Close contact between a phosphate linkage and a Co(III) center may contribute to the high catalytic effects. Moreover, the unique gel-like<sup>26</sup> microenvironment of PCD may exert favorable effects on the reactivity of CoCyc in the reaction proceeding through the mechanism of 2. In this regard, remarkable acceleration<sup>27</sup> of an RNA model reaction by organic solvents is noteworthy. It is also possible that the double-helical structure of the DNA duplex bound onto the resin is easily deformed and, thus, becomes more susceptible to hydrolysis.

Activation of the catalytic group is important in designing artificial enzymes. The remarkable activation of the CoCyc unit upon attachment to the PCD resin is expected to lead to design of effective artificial DNA hydrolases. For example, catalysts for sequence-selective hydrolytic cleavage of DNA molecules may be designed by incorporating extra catalytic elements in the vicinity of the CoCyc moiety on the resin surface. As mentioned above, the protein-cleaving activity of Cu(II)Cyc was greatly improved when Cu(II)Cyc was attached to PCD.<sup>15</sup> Enhancement of DNA hydrolyzing activity of CoCyc upon attachment to PCD in the present study suggests that PCD can be used as the backbone for immobile artificial metalloenzymes with highly activated metallo-catalytic centers.

### Acknowledgements

This work was supported by Center for Molecular Catalysis and Artzyme Biotech Corporation. CHK was the recipient of BK21 postdoctoral fellowship.

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- For the CoCyc (**1a**)-catalyzed reactions, the reactions were quenched as described in the literature prior to electrophoretic analysis: Geue, R. J.; Sargeson, A. M.; Wijesekera, R. *Aust. J. Chem.* **1993**, *46*, 1021.
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- As analyzed with **3**, only a minor portion of CoCyc moiety can act as the catalytic groups even among those exposed on the resin surface. Then, the degree of activation should be considerably greater than that estimated in the text.
- The rates for hydrolysis of supercoiled pBR 322 DNA by **1b–d** were much faster than that by **1a** at low catalyst concentrations ( $< 1$  mM) as complexation of DNA to the catalyst was facilitated by the cationic substituents.<sup>9</sup> When the DNA was fully complexed to the catalyst at the catalyst concentrations of 3–5 mM (pH 7 and 37°C), however, rates for **1a–d** were almost identical. For the PCD-based catalyst of the present study, the rate is several hundred-times faster than that of **1a** at  $C_o = 4$  mM (Fig. 2). This indicates that the enhancement in the catalytic activity of CoCyc upon attachment to PCD is not simply due to facilitated complexation of the substrate.
- Mechanism of **2** requires that one of the Co(III)-bound water be unionized and the other ionized, predicting a bell-shaped pH profile for the rate. The bell-shaped pH profiles of Figure 3 suggest that the  $pK_a$  values of water molecules bound to the Co(III) centers attached to the resin are 5–7.
- The linear dependence of  $k_o$  on  $C_o$  (Fig. 2) indicates that complexation of DNA to the resin is not considerable under the reaction conditions: the linearity of the plot up to  $C_o = 4$  mM shows that  $K_m > 4$  mM. For **1a** and **1b** ( $n = 6$ ),  $K_m$  was 0.98 mM and 0.19 mM, respectively, for the hydrolysis of supercoiled pBR 322 at pH 7 and 37°C.<sup>9</sup> Complexation of supercoiled DNA to the PCD containing multiple **1a**, therefore, is not stronger than that to **1a** dissolved in bulk water. This is attributable to the rugged nature of the resin surface which hampers multiple electrostatic interaction between the resin and the DNA and/or microenvironment of the resin unfavorable for the DNA complexation.
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