# Synthesis and Properties of Cationic Polynucleotide Analog Binding RNase A

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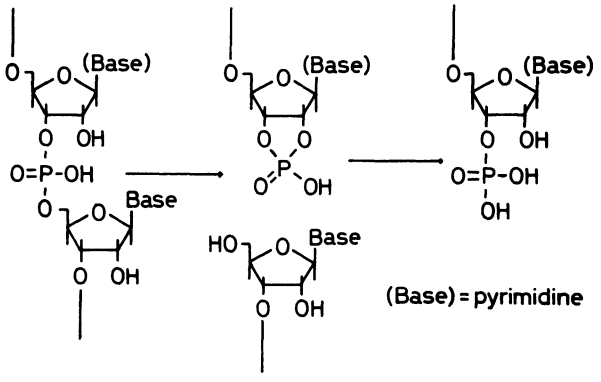
A new type of cationic polynucleotide analog binding RNase A and its related compounds were synthesized from an insoluble cross-linked poly(4-vinylpyridine). The cross-linked poly(4-vinylpyridine) was converted into an aldehyde derivative by the reaction with cyanogen bromide. The aldehyde derivative was used for the binding of RNase A and 9-(2-aminoethyl)adenine through their amino groups. The cationic adenine polynuleotide analog binding RNase A favorably interacted with nucleotides containing uracil base through the base-specific interactions. This analog showed the considerable selectivity in the hydrolysis of uridine polynucleotide owing to the template effect.

Several cationic polynucleotide analogs have been synthesized[1-3] and their interactive properties with nucleic acid bases,[4] nucleotides,[2,3,5] polynucleotides,[4] and nucleotide analogs[6,7] have also been investigated. In these studies, the mode of interactions was predominantly the base-base stacking. On the other hand, the complementary base-base interaction was pronounced in the interactions between polynucleotides and mononucleotide analogs with positive charge.[8] It was deduced that the ionic field originating from polynucleotide or its analogs makes a large contribution to the base-base interaction: the catinic field contributes to the base stacking interaction, on the other hand, the anionic one contributes to the hydrogen bonding in the complementary base-base interaction. This evidence for the contribution of the ionic field has been found in the interaction of nucleotides and nucleotide analogs in the presence of polyelectrolytes.[6,7] The mode of the base-base interaction was also affected by the hydrophobic polymer domain. The cross-linked cationic polynucleotide analogs have a tendency to interact with the complementary nucleotide.[5] The template-directed syntheses of oligonucleotides were carried out and homogeneous oligonucleotides, in which the base units are complementary to those in the polymeric "template," were found in considerable amounts, whereas scarcely any homogeneous oligomers consisting of the other nucleotide units were observed.[9]

It is expected that cross-linked cationic polynucleotide analogs, which played a role of the template, would have the ability to control the substrate specificity of enzymes which hydrolyze nucleic acids. RNase A is specific for both uracil and cytosine ribonucleotide linkages, *i.e.*, those cleaving cyclic 2′,3′-pyrimidine nucleotide residues either singly or at the termination of purine nucleotide chains[10] (Scheme 1). In order to control the substrate specificity or base specificity of RNase A, we have first time tried to bind RNase A on a cross-linked cationic polynucleotide analog. The analog consists of adenine base which is the complementary base to uracil. In this paper we describe the catalytic properties of the cationic polynucleotide analog binding RNase A in the hydrolysis of pyrimidine nucleotide linkages. This analog shows the consider able selectivity in the hydrolysis of uridine polynucleotide.




Scheme 1.

## Results and Discussion

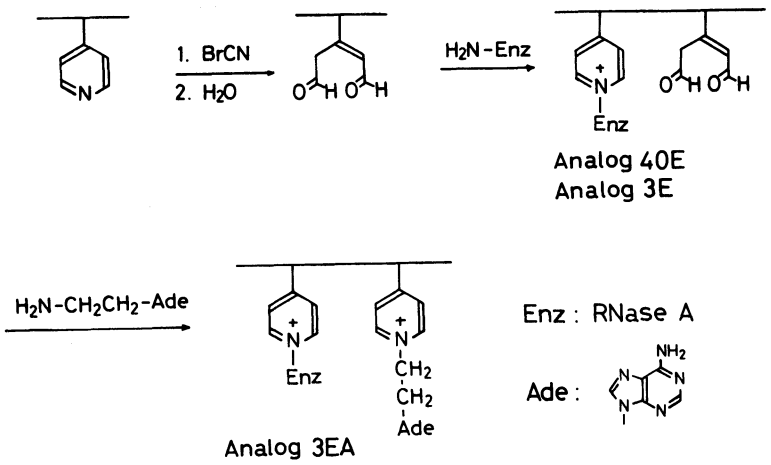
A new type of cationic polynucleotide analog binding RNase A[11] and its related compounds were synthesized from an insoluble cross-linked poly(4-vinylpyridine), according to the following scheme (Scheme 2). The cross-linked poly(4-vinylpyridine) was converted to an aldehyde derivative by the reaction with cyanogen bromide.[12] The aldehyde derivative formed was used for the binding of RNase A through its ε-amino groups and of 9-(2-aminoethyl)adenine. Excess formyl groups were reduced with borohydrides. The amounts of RNase A and adenine residue bound to poly(4-vinylpyridine), together with their abbreviations, were shown in Table 1.

In order to examine the interactive property or the template effect of the cationic polynucleotide analog binding RNase A, Analog 3EA, the affinity of the pyrimidine nucleotides with Analog 3EA and its related

Table 1. The amounts of RNase A and adenine residue bound to poly(4-vinylpyridine)

| | RNase A | Adenine residue |
|---|---|---|
| | μmol/g of dry Analog | mmol/g of dry Analog |
| Analog 40E[a] | 0.47 | — |
| Analog 3E[b] | 0.29 | — |
| Analog 3EA[b] | 0.29 | 0.34 |

Cationic polynucleotide analog binding RNase A and its related compounds were synthesized from; a) 40% cross-linked poly(4-vinylpyridine) and b) 3% cross-linked poly(4-vinylpyridine).




Scheme 2.

TABLE 2. ADSORPTION OF PYRIMIDINE NUCLEOSIDE 2′,3′-CYCLIC PHOSPHATE[a] ON ANALOG[b]

| System | $\frac{\text{Adsorbed nucleotide}}{\text{Initial nucleotide}} \times 100$ |
|---|---|
| Analog 3E–Cp> | 0.2 |
| Analog 3E–Up> | 2.5 |
| Analog 3EA–Cp> | 4.4 |
| Analog 3EA–Up> | 8.1 |

a) Nucleotide, $1.7 \times 10^{-2}$ mol dm$^{-3}$. b) Analog, 8 mg.

compound, Analog 3E, was investigated. A solution of uridine 2′,3′-cyclic phosphate (Up>) or cytidine 2′,3′-cyclic phosphate (Cp>) was added to the analogs and then the mixture was shaken. The amount of adsorbed nucleotides on the analog was determined by UV absorbance of the supernatant. The results are shown in Table 2. Analog 3EA favorably interacts with Up>, which is caused by the specific interaction of bases.

Hydrolyses of pyrimidine nucleoside 2′,3′-cyclic phosphates in the presence of the cationic polynucleotide analog binding RNase A, Analog 3EA, its related compounds, Analog 40E, Analog 3E, and the native RNase A were carried out at 25 °C in 0.1 M† phosphate buffer (pH 7.0). The steady-state kinetic parameters are summarized in Table 3. When RNase A was immobilized on the 3% cross-linked poly(vinylpyridine), the apparent $K_m$ values of Analog 3E in the hydrolyses of the both substrates were smaller than those of the native RNase A. It is suggested that the analog promotes the binding of the substrates. Analog 3E having a positive charge which is resulted from the immobilization of RNase A interacts with the anionic substrates through the coulombic attractive force. This interaction may promote the complex formation between the enzyme and the anionic substrates. On the contrary, by the immobilization of RNase A on the highly cross-linked poly(vinylpyridine) (40%), Analog 40E, the interaction with the substrates was slightly lower than that of the native RNase A. The $k_{cat}$ and $V_{max}$ values of Analog 3E in the hydrolyses of the both substrates were considerably smaller than those of the native RNase A. This means that the catalytic activity of RNase A was reduced by the immobilization on the cross-linked poly(4-vinylpyridine). That $k_{cat}$ and $V_{max}$ of Analog 40E in the hydrolysis of Cp> was close to those of Analog 3E shows the less effect of the degree of cross-linking on the catalytic activity. Of the cationic polynucleotide analog binding RNase A, Analog 3EA, the adenine residue showed no significant effect on the $K_m$, $k_{cat}$, and $V_{max}$ values in the hydrolyses of not only Cp>, but also Up>. The results that Analog 3EA adsorbs the larger amount of Up> and shows no specificity in the hydrolysis of Up> indicate that the substrates are mainly adsorbed on Analog 3EA at the adenine residues which are relatively remote from the active center of the immobilized enzyme RNase A.

Hydrolyses of 3′-5′ diuridine monophosphate (UpU)

TABLE 3. THE STEADY-STATE KINETIC PARAMETERS IN THE HYDROLYSIS OF PYRIMIDINE NUCLEOSIDE 2′,3′-CYCLIC PHOSPHATE

| Enzyme | Substrate | $K_m$ / mol dm$^{-3}$ | $k_{cat}$ / s$^{-1}$ | $V_{max}$ / mol dm$^{-3}$ s$^{-1}$ |
|---|---|---|---|---|
| RNase A | Cp> | $1.4 \times 10^{-2}$ | $2.6 \times 10^{-2}$ | $1.0 \times 10^{-2}$ |
| RNase A | Up> | $1.1 \times 10^{-2}$ | $8.8 \times 10$ | $3.5 \times 10^{-3}$ |
| Analog 40E | Cp> | $4.3 \times 10^{-2}$ | $3.0 \times 10^{-2}$ | $2.7 \times 10^{-6}$ |
| Analog 3E | Cp> | $4.3 \times 10^{-3}$ | $3.5 \times 10^{-2}$ | $1.4 \times 10^{-6}$ |
| Analog 3E | Up> | $2.6 \times 10^{-3}$ | $6.4 \times 10^{-3}$ | $2.6 \times 10^{-7}$ |
| Analog 3EA | Cp> | $8.8 \times 10^{-3}$ | $3.8 \times 10^{-2}$ | $1.5 \times 10^{-6}$ |
| Analog 3EA | Up> | $5.2 \times 10^{-3}$ | $6.1 \times 10^{-3}$ | $2.4 \times 10^{-7}$ |

† 1 M=1 mol dm$^{-3}$.

Table 4. Hydrolyses of dinucleotides[a] by Analogs 3E[b] and 3EA[b]

| | Yield of product / % | | | |
|---|---|---|---|---|
| | C | Cp | Cp> | CpC |
| Analog 3E–CpC | 51.3 | 26.7 | 22.0 | Trace |
| Analog 3EA–CpC | 47.0 | 22.7 | 30.3 | |
| | Yield of product / % | | | |
| | U | Up | Up> | UpU |
| Analog 3E–UpU | 19.0 | 2.1 | 15.6 | 63.4 |
| Analog 3EA–UpU | 10.0 | 0.8 | 8.8 | 80.3 |

Reactions were carried out at 25 °C for 2 h.
a) Dinucleotide, $2.3\times10^{-3}$ mol dm$^{-3}$. b) Enzyme on Analog, $1.6\times10^{-5}$ mol dm$^{-3}$.

and 3′-5′ dicytidine monophosphate (CpC) in the presence of Analogs 3EA and 3E were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.0) for 2 h. The hydrolyzed products were analyzed by HPLC. The results are shown in Table 4. CpC was hydrolyzed in the presence of both Analog 3EA and Analog 3E to the almost similar composition of the products, Cp>, Cp, and C. Similary, the distribution of the products in the hydrolysis of UpU by Analog 3EA and Analog 3E was not significantly different. These results show that Analog 3EA has no specificity in the hydrolyses of both UpU and CpC.

This feature was drastically changed in the hydrolysis of Poly U and Poly C as substrates. The results are shown in Table 5. Poly C was hydrolyzed in the presence of Analog 3EA to Cp, Cp>, CpCp, and CpCpCp. The amounts of CpCp and CpCpCp were slightly larger than those in the case of Analog 3E. On the other hand, the amounts of the products, UpUp> and UpUpUp, in the hydrolysis of Poly U by Analog 3EA were one-half to one-forth of those by Analog 3E. That is, the hydrolysis rate of Poly U by Analog 3EA is much higher than by Analog 3E. These results imply that there is no effect of adenine residue on the hydrolysis of Poly C but a considerable effect on Poly U hydrolysis. This effect is considered as follows; Poly U adsorbs on Analog 3EA through the complementary base-base interaction and it approaches to the active center of RNase A bound to Resin 3EA, for the polynucleotides having a long chain are easy to approach to the active center of the enzyme, even though the adsorption site of substrates is apart from the active center. The present study shows that the new type of cationic polynucleotide analog binding RNase A controls the substrate specificity of enzyme through the template effect.

## Experimental

*Materials.* RNase A (Bovine Pancrease) was perchased from Sigma Chemical Co. 9-(2-aminoethyl)adenine was synthesized by the reported method.[13] 3% and 40% cross-linked poly (4-vinylpyridine) was synthesized by the copolymerization of 4-vinylpyridine with divinylbenzene. Up>, Cp>, UpU, CpC, Poly U (M. W.=10000) and Poly C (M. W.=10000) were obtained from Sigma Chemical Co.

*Preparation of Cationic Polynucleotide Analog Binding RNase A and Its Related Compounds.* *a) Analog 40E*: 3 g of 40% cross-linked poly(4-vinylpyridine) was reacted with 1.5 g of cyanogen bromide in 10 cm$^3$ of anhydrous dioxane at room temperature for 15 min. Water was then added and the mixture was stirred for 90 min at room temperature. The gel was filtered and washed with cold water. 1.5 g of the activated gel was stirred overnight in a solution of RNase A (20 mg) in 4 cm$^3$ of 0.05 M phosphate buffer, pH 5.0, at 10—12 °C. The gel was then treated with 1% $NaBH_4$ at room temperature for 10 min and then washed with the phosphate buffer.

*b) Analog 3E*: 2.6 g of 3% cross-linked poly(4-vinylpyridine) was reacted with 2.5 g of cyanogen bromide in 10 cm$^3$ of anhydrous dioxane at room temperature for 15 min. After the treatment with water for 60 min, the gel was washed with cold water. 0.5 g of the activated gel was stirred overnight in a solution of RNase A (23 mg) in 2 cm$^3$ of 0.05 M phosphate buffer, pH 5.0, at 4 °C. The gel was then treated with 1% $NaBH_4$ at room temperature for 10 min and then washed with the phosphate buffer.

*c) Analog 3EA*: $NaBH_4$-untreated Analog 3E was reacted with 50 mg of 9-(2-aminoethyl)adenine in the same buffer at 4 °C for overnight. Then, the gel was treated with 1% $NaBH_4$ at room temperature for 10 min and then washed with the phosphate buffer.

*The Amount of RNase A and Adenine Residue Bound to Polynucleotide Analog.* The amount of RNase A on Analog was determined as follows. Analog was treated with 6 M HCl at 50 °C for 6 h and the amount of released tyrosine was measured at 294 nm ($\varepsilon=2.39\times10^3$). Calculation was carried out on the fact that RNase A (M. W.=13700) contains 6 tyrosine residues.[14] The amount of adenine residue on Analog 3EA was determined from the amount of the unreacted 9-(2-aminoethyl)adenine in the washing of the reaction mixture.

*Adsorption of the Substrate on Analog.* A solution of the substrate ($1.7\times10^{-5}$ mol) in 0.1 M phosphate buffer, pH 7.0,

Table 5. Hydrolyses of polynucleotides[a] by Analogs 3E[b] and 3EA[b]

| | Yield of product / % | | | | |
|---|---|---|---|---|---|
| | Cp | Cp> | CpCp | CpCp> | CpCpCp |
| Analog 3E–Poly C | 53.6 | 38.2 | 4.9 | Trace | 4.0 |
| Analog 3EA–Poly C | 43.4 | 42.3 | 5.7 | Trace | 8.6 |
| | Yield of product / % | | | | |
| | Up | Up> | UpUp | UpUp> | UpUpUp |
| Analog 3E–Poly U | 20.3 | 37.0 | 10.7 | 24.6 | 7.7 |
| Analog 3EA–Poly U | 25.8 | 47.4 | 11.0 | 13.6 | 1.9 |

Reactions were carried out at 25 °C for 2 h. a) Polynucleotide, $3.7\times10^{-3}$ mol dm$^{-3}$. b) Enzyme on Analog, $1.6\times10^{-5}$ mol dm$^{-3}$.

(1.0 $cm^3$) was added to Analog (8 mg). The difference between the initial and the final (5 min after) optical densities of the supernatant was measured by UV spectroscopy at 260 nm. Thus, the apparent adsorption of the substrate on Analog was estimated.

*Activity of RNase A Bound to Polynucleotide Analog.* Up>, Cp>, UpU, CpC, Poly U, and Poly C were used for substrates. Hydrolyses of these substrates in the presence of Analog was carried out in 0.1 M phosphate buffer, pH 7.0, at 25 °C. Analysis of hydrolytic products was done on a HPLC(Partisil 10 SAX column; 0.001 M $KH_2PO_4$—0.3 M $KH_2PO_4$ containing 10% EtOH, a linear gradient).

*a) Experiment in Table 3*: Hydrolysis was carried out in a mixture of substrate (Up> and Cp>) ($2.2\times10^{-8}$—$4.1\times10^{-7}$ mol), 0.1 M phosphate buffer pH 7.0 (0.1 $cm^3$), and Analog (15 mg for Analog 40E, 30 mg for Analogs 3E and 3EA). After incubation at 25 °C for 30 min, the products were filtered by Milipore Filter (Type HA, 0.45 μm) and analyzed by HPLC. Control experiment for soluble RNase A was done in a mixture of the substrates ($2.2\times10^{-8}$—$4.1\times10^{-7}$ mol), 0.1 M phophate buffer pH 7.0 (0.1 $cm^3$), and the enzyme (final concentration, $4.0\times10^{-5}$ mol $dm^{-3}$) at 25 °C for 5 min. Analysis of the products were the same as for Analog. Thus, the kinetic constants were determined by the Michaelis-Menten method.

*b) Experiments in Tables 4 and 5*: Hydrolysis was carried out in a mixture of substrates ($2.3\times10^{-3}$ mol for dinucleotide monophosphate, $3.7\times10^{-3}$ mol for polynucleotide), 0.1 M phophate buffer, pH 7.0, (0.1 $cm^3$), and Analog (5 mg). After incubation at 25 °C for 2 h, the products were filtered by Milipore Filter (Type HA, 0.45 μm) and analyzed by HPLC.

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