Polynucleotides. XXVI. ¹⁾ Complex formation of polynucleotides derived from formycin and laurusin with cyclonucleoside oligonucleotides.

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ABSTRACT

Poly(formycin phosphate) and poly(laurusin phosphate) were synthesized by polymerizing formycin and laurusin 5'-diphosphate by means of *E. coli* polynucleotide phosphorylase. The complex formation of these polynucleotides with cyclonucleoside polynucleotides were investigated. While poly(formycin phosphate) did not form the complex with an octanucleotide of 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil, poly(laurusin phosphate) did form a 1 : 1 complex with octanucleotide of 8,2'-anhydro-8-mercapto-9- β -D-arabinofuranosyladenine in the presence of 0.15M Na ion at neutrality and 3⁰. CD spectrum of this complex showed a couple of a trough at 286 nm and a peak at 262 nm. This fact suggests that the complex has a left-handed helical conformation, which is opposite to the natural double helical polynucleotides. The cause of this phenomenon was discussed in connection with the complex of cyclonucleoside oligonucleotides.

We have previously shown that oligonucleotides of 8,2'-anhydro-8-mercapto-9- β -D-arabinofuranosyladenine (A^B) (I) had a single-stranded ^{2,3)} structure, in which bases stacked along the left-handed helical axis, and a double-stranded complex of the same helical conformation with 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil (U^O) (II) oligonucleotides. ⁴⁾ The cause of this unusual helical conformation of left-handed helix was thought to be fixation of adenine moieties in cyclonucleoside oligomers to the torsion angle ⁵⁾ $\phi_{CN} -120^{\circ}$. ³⁾

Structural formula of 8,2'-anhydro-8-mercapto-9-β-D-arabinofuranosyl-adenine (A^S) (I), 6,2'-anhydro-6-oxy-1-β-D-arabinofuranosyluracil (U^O) (II), formycin (F) (III), and laurusin (L) (IV)

The antibiotic formycin⁶⁾ (F) (III) has long been known to possess a syn conformation according to the X-ray crystallographical study of its hydrochloride.⁷⁾ Recently, however, Prusiner et al.⁸⁾ reported that formycin had a torsion angle $\phi_{\text{CN}} -109.8^\circ$ in a hydrated form. From the study of Ward et al.⁹⁾ it was deduced that bases in the single-stranded poly (F) existed in syn conformation, whereas in the double-stranded poly (F)·poly (U) complex they existed in anti form. This flexibility of the torsion angle may be ascribed to the unique structure of formycin, in which usual nucleosidic linkage was replaced by a C-C bond. If this flexible conformation holds good, poly (F) should form a complex with (pU^O)_n, which has the torsion angle in the range of syn-anti boundary region.

Laurusin¹⁰⁾ (L) (IV) is a deaminated form of formycin, i.e. an inosine analog, and it should form the complex with (pA^S)_n in the appropriate condition as was observed in the case of poly (I) and poly (A).¹¹⁾

Formycin 5'-monophosphate was synthesized by the phosphorylation of formycin with phosphoryl chloride in trimethyl phosphate. Product was chromatographed through Dowex 1X2 column and the yield was 75%. The monophosphate was converted to morpholidate by the method of Moffatt and Khorrana¹²⁾ and allowed to react with inorganic phosphate to afford diphosphate. Purification of the diphosphate by DEAE-cellulose column chromatography gave a yield of 39%. The structure was confirmed by elemental analysis, paper chromatography, paper electrophoresis and UV absorption spectra. Poly (F) was obtained by polymerizing the diphosphate by the use of *E. coli* polynucleotide phosphorylase¹³⁾ The product was eluted from a Sephadex G-50 column in the void volume showing that this sample of poly (F) had a chain length above 50 nucleotide units.

In order to investigate the complex formation between poly (F) and $(pU^O)_8$, both polynucleotides were mixed in various molar ratios as indicated in Fig. 1. UV absorption observed in 252, 270 and 295 nm showed no inflection point indicating that there seemed to exist no complex between poly (F) and $(pU^O)_8$ at least in the presence of 0.1M sodium chloride and 0.05M sodium cacodylate at pH 7.0 and 3⁰. Heating the solution to 60⁰ and slow cooling to 0⁰ had no effect. Addition of either 1.0M sodium chloride or 10mM Mg^{2+} caused white precipitation and interfered with the measurement.

Formycin diphosphate was then deaminated with sodium nitrite in acetic acid solution to give laurusin diphosphate in a yield of 55%. The structure of the diphosphate was confirmed by elemental analysis, UV absorption, paper chromatography and paper electrophoresis. Laurusin diphosphate was polymerized by the use of polynucleotide phosphorylase. Poly(I), thus obtained, was gel-filtered with Sephadex G-50 and the chain length was confirmed as 50 nucleotide units or above. Yield was 15%.

Fig. 1. Mixing curves of poly (F) and $(pU^O)_8$ in the presence of 0.1M NaCl and 0.01M Na Cacodylate at pH 7.0 and 2° .

Fig. 2. Mixing curves of poly (L) and $(pA^S)_8$ in the presence of 0.1M NaCl and 0.05M Na Cacodylate at pH 7.0 and 2.5° .

The complex formation of poly (L) with $(pA^S)_8$ ³⁾ was then investigated by the continuous variation method. When poly (L) and $(pA^S)_8$ were mixed in various ratios as shown in Fig. 2 in the presence of 0.15M sodium chloride at pH 7.0 and 2.5°, UV absorption observed at 265 and 290 nm showed inflection points corresponding to a molar ratio 50 : 50. This result indicates that poly (L) and $(pA^S)_8$ formed a 1 : 1 complex at 2.5° as was observed in the case of poly (I) and poly (A).¹¹⁾ UV absorption spectra of the 1 : 1 complex were measured at various temperature. No melting was observed over the range from 2.5° to 76°.

CD spectrum of this complex is shown in Fig. 3. The curve shown by solid line is clearly different in shape and magnitude from that obtained by calculation of 1 : 1 mixture of poly (L) and $(pA^S)_8$ (dotted line). The CD spectrum of the complex has troughs at 286 nm ($[\theta] = -19800$) and 232 nm ($[\theta] = -17000$) and two peaks at 262 nm ($[\theta] = +23500$) and 220 nm ($[\theta] = +52000$). As was discussed previously²⁾ a couple of trough and peak centered at 274 nm could be ascribed to an exciton splitting band¹⁴⁾ ari-

Fig. 3. CD spectra of poly (L).(pA^S)₈ complex (—) and calculated sum (---) taken with 0.1M NaCl and 0.05M Na Cacodylate (pH 7.0).

sing out of the Cotton band of B-region of the monomer A^S . This type of splitting suggests the left-handedness of the stacking of bases in polynucleotide chain. Accordingly, pyrazolopyrimidine nucleosides in polynucleotide, poly (L) have to take the conformation of left-handedness in the double helical complex. If we raise the temperature of the mixing solution from 3° to 80° , no change in $[\theta]$ was observed. This fact together with UV absorption data suggests that the melting temperature (T_m) of poly (L) $\cdot$ $(pA^S)_8$ is above 80° . The cause of this thermal stability may be ascribed to the rigid conformation of A^S oligonucleotide and favorable hydrogen bondings between bases in the complex.

From these results it may be concluded that the torsion angle ϕ_{CN} of laurusin residues of poly (L) in the complex with $(pA^S)_8$ must be around -120° . Since poly (L) could form complexes with other usual polynucleotides such as poly (A) or poly (C),¹⁰⁾ the torsion angle of laurusin must be variable according to that of complementary polynucleotides. In the case of poly (L) $\cdot$ $(pA^S)_8$, the torsion angle of A^S residues is fixed by a covalent linkage and should stay at $\phi_{CN} -120^\circ$. Therefore, in order to form a rigid complex pyrazolopyrimidine rings of poly (L) must be in the range of $\phi_{CN} = -120^\circ$. By taking such a structure the helical conformation should be left-handed as found in the case of $(pA^S)_n$ ³⁾ and in $(pA^S)_8 \cdot (pU^O)_8$ ⁴⁾. The thermal stability of poly (L) $\cdot$ $(pA^S)_8$ may be ascribed to a rigid conformation of A^S residues and a favorable hydrogen bonding between A^S and pyrazolopyrimidine ring. Even for a dimer $A^S pA^S$ the melting temperature was above 80° in a standard condition²⁾ and T_m of oligomers of pA^S was even higher³⁾, presumably because of the strong stacking. If poly (L) formed hydrogen bondings with $(pA^S)_8$, this complex has to be a stable one.

The failure of observing the complex between poly (F) and $(pU^O)_8$

may be due to extremely low T_m of the desired complex. As observed in the case of poly (F)·poly (U)⁹⁾, which had fairly low T_m compared to that of poly (A)·poly (U), the backbone of poly (F) was not enough stable to make a complex with $(pU^O)_8$ in the experimental conditions.

The tentative hypothesis that polynucleotides having same (or nearly same) torsion angle of bases will form double helical complexes and if the torsion angle was in the range of 108° to 120° , the direction of the helical turn will be left-handed, was further supported.

EXPERIMENTAL

General methods

UV absorption spectra were taken with a Hitachi EPS-3T or 124 spectrophotometer and CD spectra were taken with a JASCO/ORD/UV-5 spectropolarimeter equipped with a CD attachment. Concentration of nucleotides was 1.0-2.0 OD units per ml. Cuvette of 1 cm light path length was thermostated with a JASCO low temperature apparatus. All runs were made at least twice for obtaining accurate curves and calibrated with d-10-camphorsulfonic acid. Mixing experiments were performed by a continuous variation method at concentration of 0.05-0.05 mM polynucleotides. Absorption was measured with a Hitachi 124 spectrophotometer equipped with a Komatsu Solidate SPD-H-124 thermostated cell. Temperature within the cell was measured by a Cu-constantan thermocouple. ξ and $[\theta]$ were presented as per residue value.

Paper chromatography and electrophoresis

Paper chromatography was performed on Toyo filter paper No. 51A by the descending technique in the following solvent systems : A, ethanol-1M ammonium acetate (7 : 3); B, n-butanol-acetic acid-water (5 : 2 : 3); C, isopropanol-conc. ammonia-water (7 : 1 : 2); D, n-propanol-conc. ammonia-water (55 : 10 : 35).

Paper electrophoresis was performed in 0.05M triethylammonium bicarbonate buffer (pH 7.5) at 35V/cm for 1hr on Toyo filter paper No.51A. Relative migration ratio to 5'-AMP was presented as R_{AMP} .

Formycin 5'-phosphate — Formycin (267 mg, 1 mmole) was dissolved in trimethyl phosphate (2 ml). To the solution was added a trimethyl phosphate solution (0.5 ml) containing $POCl_3$ (0.37 ml, 4 mmoles) under ice-cooling and stirring.¹⁵⁾ After 2 hrs stirring the reaction mixture was poured into ice-water, which was adjusted to pH 8 with N NaOH. The mixture was diluted with water (50 ml) and applied to a column (1.5 x 50 cm) of Dowex LX2 (formate form) resin. Elution with 0.05N formic acid at a rate of 15 ml/ 1 fraction/ 10 min gave formycin 5'-phosphate in fractions No. 35-53, which were collected (total 9050 $OD_{295}^{H^+}$ units) and desalted through a short charcoal column. Formycin 5'-phosphate was obtained in a yield of 7400 $OD_{295}^{OH^-}$ units (75 %). Paper chromatography : Rf(B) 0.10 (formycin 0.32), Rf(C) 0.05 (formycin 0.39). Paper electrophoresis : R_{AMP} 0.99. When this material (5 OD_{295} units) was incubated with crude snake venom (5'-nucleotidase) (10 mg/ml, 20 λ) and 1M ammonium acetate (20 λ) in a incubation mixture (100 λ) at 37° for 4.5 hrs, dephosphorylation proceeded completely. This showed that the product was formycin 5'-monophosphate.

Formycin 5'-diphosphate — Formycin 5'-monophosphate (9000 OD_{295} units, 0.9 mmole) was dissolved in water (9 ml) and t-butanol (9 ml). After adding morpholine (0.29 ml, 3.6 mmoles) to the mixture, a n-butanol(15 ml) solution of DCC (0.7 g, 3.6 mmoles) was added dropwise under refluxing. The refluxing was maintained for 4 hrs and total conversion of the 5'-phosphate to the morpholidate was confirmed by paper electrophoresis. The mixture was filtered and dicyclohexylurea was washed with t-butanol. The filtrate and washings were combined and t-butanol was evaporated in

vacuo. Water layer was extracted with ether and evaporated to dryness in vacuo. Formycin 5'-phosphoromorpholidate was obtained in a yield of 7400 OD₂₉₅ units. The morpholidate was made anhydrous by repeated evaporation with added pyridine. Phosphoric acid (85%, 0.245 ml, 3.6 mmoles) and tri-n-butylamine (0.86 ml, 3.6 mmoles) were combined and made anhydrous with pyridine. The both residues were taken up in pyridine (2 ml) and evaporated again. The final residue was dissolved in pyridine (9 ml) and kept at room temperature for 4 days under exclusion of moisture. The solvent was evaporated in vacuo, the residue was dissolved in water, and it was adjusted to pH 4 with N HCl. The solution was applied to a charcoal column for desalting and effluents were applied to a column (3 x 45 cm) of DEAE-cellulose (carbonate form). Elution was performed with water (3 l.) and 0.3M triethylammonium bicarbonate buffer (3 l.) by a linear gradient technique in a rate of 15 ml/fraction/10 min. Fractions No. 240-280 were collected and desalted through a charcoal column to give formycin 5'-diphosphate (2600 OD₂₉₅ units). Fractions No. 215-239 (1130 OD₂₉₅ units) were rechromatographed through a column (1 x 55 cm) of DEAE-cellulose (bicarbonate form) to separate diphosphate from formycin 5',5'-pyrophosphate. Elution with 0.1M triethylammonium bicarbonate (1.5 l.) and 0.2M triethylammonium bicarbonate (1.5 l.) gave 950 OD₂₉₅ units of formycin 5'-diphosphate. Total yield was 39%. UV : $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 295 nm. Paper chromatography: R_f(B) 0.02. Paper electrophoresis : R_{AMP} 1.20 (ADP, 1.15). Ratio of base-acid labile phosphate-total phosphate was 1.0 : 1.02 : 2.01.

Laurusin 5'-diphosphate — Formycin 5'-diphosphate (950 OD₂₉₅ units, 0.095 mmole) and sodium nitrite (120 mg) was dissolved in water (1 ml). Acetic acid (0.065 ml) was added to the solution, which was kept at room temperature for 4 hrs and at 0-5° for further 10 hrs. The solution was

adsorbed on charcoal, which was washed thoroughly with water. Elution with 50% ethanol containing 2% conc. ammonia and evaporation of the effluents gave crude laurusin 5'-diphosphate. The residue was dissolved in water (5 ml) and applied to a column (0.6 x 14 cm) of Dowex 1X2 (chloride form). Elution was performed with 0.075 N HCl (0.5 l.) and 0.075N HCl + 0.1M NaCl (0.5 l.) in a linear gradient technique. The flow rate was 5 ml/fraction/10 min. Fractions No. 115-145 were collected and desalted through a charcoal column. Yield of laurusin 5'-diphosphate was 434 OD₂₈₀ units (55 %). UV : $\lambda_{\text{max}}^{\text{pH} 7.0}$ 218, 280 nm; $\lambda_{\text{max}}^{\text{pH} 1}$ 221.5, 275.5 nm; $\lambda_{\text{max}}^{\text{pH} 13}$ 228, 291 nm. Paper chromatography : R_f(A) 0.14, R_f(D) 0.40. Paper electrophoresis : R_{AMP} 1.28. Ratio of base-labile phosphate-total phosphate was 1.0 : 1.01 : 1.98 (theor. 1 : 1 : 2).

Poly (F) --- Formycin 5'-diphosphate (1000 OD₂₉₅ units, 100 μ moles), MgCl₂ (40 μ moles), Tris-HCl (pH 8.2, 1000 μ moles) and polynucleotide phosphorylase (32 units) were incubated in a incubation mixture (20 ml) at 37° for 4 days. The mixture was deproteinized by extraction with CHCl₃-isoamylalcohol (3 : 1, vol/vol) mixture and the water layer was concentrated in a collodion bag. Contents of the bag were applied to a column (1.6 x 120 cm) of a Sephadex G-50 and eluted with water. Fractions (5 ml each) were collected every 30 min. The polynucleotide was eluted in the void column. Effluents were lyophilized to give 92 OD₂₉₅ units (12 %) of poly (F). UV taken with 0.1M NaCl at pH 7.0 : λ_{max} 294.5 nm (ϵ 8060), 289 nm (ϵ 8000), 230 (shoulder), 306 (shoulder).

Poly (L) --- Laurusin 5'-diphosphate (200 OD₂₈₀ units, 23 μ moles), MgCl₂ (7 μ moles), Tris-HCl (pH 9.0, 100 μ moles) and polynucleotide phosphorylase (8 units) were adjusted to 3 ml with water and incubated at 37° for 47 hrs. The incubation mixture was extracted with CHCl₃-isoamylalcohol (3 : 1, vol/vol) and applied to the ultrafiltration in a collodion bag. Con-

tents of the bag were applied to a column (1.6 x 120 cm) of Sephadex G-50. Fractions (5ml each) eluted at the void volume (No. 16-19) were collected and evaporated in vacuo. Poly (L) was obtained in a yield of 23 OD₂₈₃ units (15 %). UV taken with 0.15M NaCl at pH 7.0 and 15° : λ max 285 nm (ϵ 6600), 277 nm (shoulder), 296 nm (shoulder).

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