Volume 4 Number 8 August 1977 Nucleic Acids Research

# Polynucleotides. XLV[1] Synthesis and properties of poly(2'-azido-2'-deoxyinosinic acid)

Toshikazu Fukui, Nubuko Kakiuchi and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

Received 14 April 1977

ABSTRACT

Poly (2'-azido-2'-deoxyinosinic acid), [poly (Iz)], was synthesized from 2'-azido-2'-deoxyinosine diphosphate by the action of polynucleotide phosphorylase. Poly (Iz) has UV absorption properties similar to poly (I) and hypochromicity of 11% at 0.15M $Na^+$ and neutrality. In solutions of high $Na^+$ ion concentration, poly (Iz) forms a multi-stranded complex and its Tm at 1.0M $Na^+$ ion concentration was 43°. Upon mixing with poly (C), poly (Iz) forms a 1:1 complex having a Tm lower than that of poly (I)·poly (C) complex in the same conditions. The effect of substitution at the 2'-position of the poly (I) strand was discussed in relation to the interferon-inducing activity.

## INTRODUCTION

A number of polynucleotides containing analogues of pyrimidine and purine nucleotides have been reported.[2] The need to understand the way in which a substituent at the 2'-position of the ribose ring influences the structure and function of the polynucleotides has led to the synthesis of a variety of compounds of this type. Pyrimidine polynucleotides which contain 2'-halogeno[3], -azido[4,5], -amino[5], -methoxy[6] and -ethoxy[7] substituents have been reported[8]. We have found a new method for the synthesis of 2'-N-substituted nucleosides[9] by way of purine cyclonucleosides, which are readily available from the naturally occurring nucleosides. We have previously reported the synthesis and properties of poly (2'-azido-2'-deoxyadenylic acid)[10] as the first purine polynucleotide having 2'-N-substituents. We found that it possessed unique stacking features between adenine bases in the neutral form and in the case of complexing with poly(U). We have also synthesized poly(2'-amino-2'-deoxyadenylic acid) and studied its physical properties.[11]

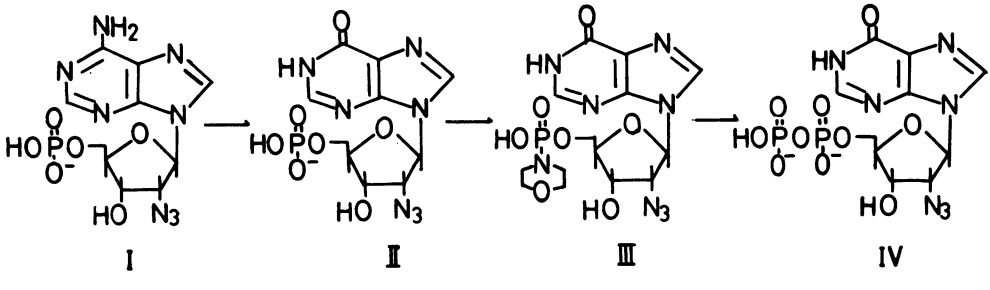
In this paper we describe the synthesis of poly(2'-azido-2'-deoxyinosinic acid)[poly(Iz)] catalyzed by E.coli polynucleotide phosphorylase together with its physical properties including UV, CD and Tm in neutral media and the results obtained on mixing with poly(C). It has been found that the introduction of the azido group at the 2'-position of 2'-deoxyinosinic acid in the polynucleotide chain led to the significant increase of the interferon inducing ability of the complex with poly(C).[12] Therefore, it seemed of interest to obtain information on the structure-function relationships of such an interferon inducer analogous to poly(I)·poly(C).

## Materials and Methods

### Synthesis of poly(2'-azido-2'-deoxyinosinic acid)

#### 2'-Azido-2'-deoxyinosine 5'-phosphate (II)

2'-Azido-2'-deoxyadenosine 5'-phosphate [10] (I, 5000 $OD_{260}$ units, 0.33 mmol) and sodium nitrite (500 mg) was dissolved in water (5 ml). Acetic acid (4 ml) was added to the solution which was kept at 37° overnight. [13] The solution was absorbed on charcoal, which was washed thoroughly with water. Elution with 50% ethanol containing 5% conc. ammonia and evaporation of the eluents gave crude 2'-azido-2'-deoxyinosine 5'-phosphate. The residue was dissolved in water and applied to a column(12x20 cm) of Dowex 1x2 (formate form). Washing with 0.175N HCOOH and elution with 0.3N HCOOH gave 2'-azido-2'-deoxyinosine 5'-phosphate (II)(3640 $OD_{250}$ units, 0.29 mmole, 87%). UV: $\lambda_{max}^{pH7.0}$ 249 nm. Paper electrophoresis: $R_{AMP}$ 1.0.



I II III IV

2'-Azido-2'-deoxyinosine 5'-diphosphate (IV)

2'-Azido-2'-deoxyinosine 5'-phosphate (3640 $OD_{250}$ units, 0.29 mmole) was dissolved in water (3 ml) and t-butanol (3 ml). After adding morpholine (0.14 ml, 1.2 mmoles) to the mixture, a t-butanol (4.5 ml) solution of DCC (252 mg, 1.2 mmoles) was added dropwise under reflux.[14] After refluxing for 4 hr, the mixture was evaporated in vacuo and the residue was equilibrated in an $H_2O$-ether (1:1) mixture. Insoluble material was filtered off and the aqueous layer was separated and evaporated. The residue was azeotropically dried with pyridine several times to give 2'-azido-2'-deoxyinosine 5'-phosphoromorpholidate (III) as a hard syrup. To the residue inorganic phosphate (85% aqueous solution 0.07 ml, 1.03 mmole), which was previously dried by evaporation with pyridine together with tri-n-butylamine (0.24 ml, 1.0 mmole) and dissolved in pyridine (2 ml), was added. The reaction mixture was kept at 30° for 2 days. The reaction mixture was evaporated in vacuo, the residue dissolved in water, and applied to a column of charcoal. The column was washed with water and eluted with 50% ethanol containing 5% conc. ammonia. Eluents were concentrated and applied to a column (1.7x20 cm) of DEAE-Sephadex A25 (bicarbonate form). Elution was performed with 0-0.25M triethylammonium bicarbonate buffer (pH 7.5, total 4 l) in a linear gradient and 20 ml fractions were collected. Fractions No 161-200 were pooled and evaporated. 2'-Azido-2'-deoxyIDP (1575 $OD_{250}$ units, 43%) was obtained as an amorphous powder. Paper electrophoresis: $R_{AMP}$ 1.22. Ratio of base: labile phosphate: total phosphate, 1.0:1.1:2.1.

Polymerization of 2'-azido-2'-deoxyinosine 5'-diphosphate

The polymerization mixture (12.5 ml) contained Tris-HCl (pH 8.5, 80 mM), $MnCl_2$ (2 mM), 2'-azido-2'-deoxyIDP (4 mM), and 2.4 units of polynucleotide phosphorylase per milliliter of solution. Incubation was performed at 37°. Progress of the reaction was followed by inorganic phosphate analyses [15] on aliquots (0.25 ml) removed at various time intervals. (Fig 1) After 17 hrs the mixture was deproteinised with isoamylalcohol-$CHCl_3$ (1:3 v/v). The organic phases were

combined and extracted with water. The resulting aqueous solutions were combined and lyophilized to dryness. The residue was dissolved in water and applied to a Sephadex G50 column (1.7x110 cm) which was eluted with water. The polymer was eluted in the void volumn. The appropriate fractions containing polynucleotide were combined and dialyzed against 5 l of 0.01M EDTA-0.01M Tris-HCl (pH 7.0) and then against 5 l of water. The resulting aqueous solution was lyophilized. Usually IzDP (50 $\mu$moles) was polymerized as described above. After purification, the yield of poly(Iz) was 75 $OD_{250}$ units (7.04 $\mu$moles, 14%). UV: $\lambda$ max 247 nm ($\varepsilon$ 10,950) at 18° in 0.10M NaCl-0.05M Na cacodylate (pH 7.0).

Physical Measurements

UV spectra were taken with a Hitachi 124 or 200 spectrophotometer in the same conditions described above.
The extinction coefficient of poly(Iz) was determined by inorganic phosphate analysis after digestion with acid as described by Howard et al. [16] An average value for the three Pi determinations gave 10,950 at $\lambda$ max for poly (Iz).
CD spectra were taken with a JASCO ORD-UV-5 spectrometer equipped with a CD attachment in the presence of 0.1M NaCl and 0.05M Na cacodylate (pH 7.0). Melting temperature was measured with a Hitachi spectrometer equipped with a thermostated cell. The temperature inside the cell was measured with Sibaura thermister Model MGB-III type 218. Mixing curves were obtained by measuring the absorbance of mixtures which contained 0.04mM total concentration of poly(Iz) and poly(C) in the ratios indicated in Fig. 5. Salt concentration was 0.1M NaCl and pH was adjusted to 7.0 with 0.05M Na cacodylate.

## RESULTS AND DISCUSSION

IzDP was a substrate for polynucleotide phosphorylase from E.coli on incubation at pH 8.5 in the presence of $Mn^{2+}$ ions. Fig.1 shows the time course. As in the case of AzDP,[10] IzDP was a very poor substrate. The organic phosphate liberated was 40% after 17 hrs incubation, but the isolated yield of poly(Iz) was only 14%.

UV and CD spectra of poly(Iz)

UV absorption of poly(Iz) is shown in Fig 2a. Poly(Iz)

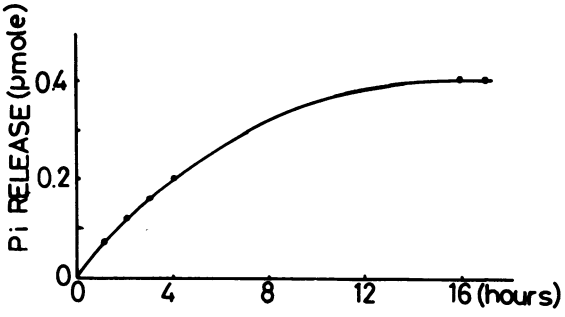



Fig. 1 **Time course of polymerization** of 2'-azido-2'-deoxy-inosine 5'-diphosphate

showed $\lambda$ max 247 nm ($\varepsilon$ 10,950) as compared with $\lambda$ max 249 nm ($\varepsilon$ 12,500) for IzMP. A blue shift of 2 nm of the $\lambda$ max compared to that of the monomer was observed. The magnitude of this shift was the same as that observed for poly(I). Hypochromicity at neutrality in the presence of 0.15M $Na^+$ was calculated as 11% assuming $\varepsilon$ of the monomer equal to 12,500.

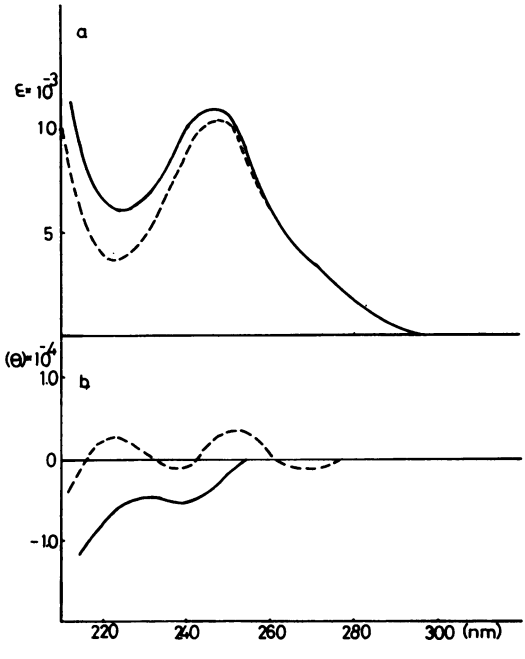



Fig 2 U.V. and C.D. spectra of poly(Iz) and poly(I) in neutral solution containing 0.15M $Na^+$.
—— poly(Iz), ---- poly(I)

This hypochromicity is smaller than that of poly(I) (15%) under the same conditions.

The CD spectra of poly(Iz) and poly(I) at 8° in 0.10M MgCl-0.05M Na cacodylate (pH 7.0) are shown in Fig. 2b. The CD spectrum of poly(Iz) did not show any positive band in the UV region (210-320 nm). A trough appeared at around 240 nm ([θ]-5,300). This suggests,a stacked random coil structure which was less stable than poly(I) for poly(Iz) in the neutral solution in the presence of 0.15M $Na^+$.

According to Rich [17] poly(I) associates under appropriate environmental conditions to form a three-stranded helical complex with three hydrogen bonds involved in stabilization of each hypoxanthine triplet. Recently Arnott et al, presented a quadruplet structure for this complex. [18]

As shown in Fig.3, the optical density of poly(Iz) was decreased by changing the solvent from 0.05M to 0.95M $Na^+$ ion concentration. On going from 0.05M to 0.95M, the optical density at 248 nm was descreased by 19%. This fact suggests that, while poly(Iz) exists as a random coil structure in the 0.05M $Na^+$ solution, on increasing the salt concentration to 0.95M $Na^+$, the polymer associated to an ordered structure as was found in the case of poly(I).

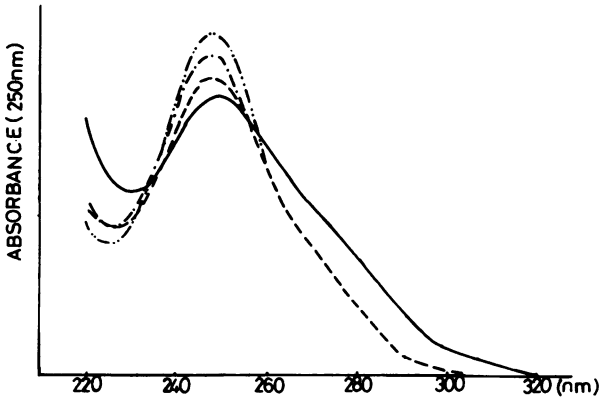



Fig.3 U.V. absorption of poly(Iz) at various $Na^+$ ion concentration. —— 0.95M, --- 0.51M, —·—·—0.35M —··—··— 0.05M

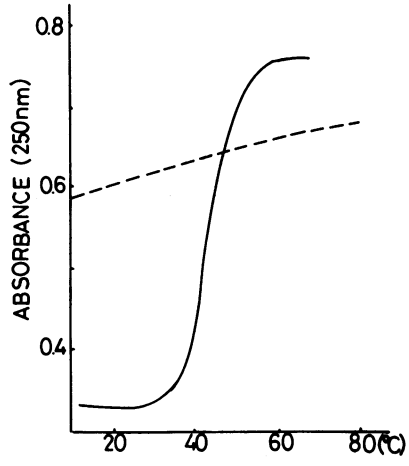



Fig.4 Tm of poly(Iz)
———0.95M $Na^+$,
-----0.15M $Na^+$

Temperature-absorbance profile

The temperature-absorbance profile of poly(Iz) in neutral conditions is shown in Fig. 4. While the UV absorption at 0.15M $Na^+$ ion increased gradually on heating from 0° to 80° without showing any steep increase indicative of cooperative melting, the curve taken with 0.95M $Na^+$ ion showed a sharp transition at 43°. The Tm (43°) for poly(Iz) is the same as the Tm (43°) of poly(I). The melting profile for poly(Iz) is somewhat less cooperative and its hyperchromicity on melting is smaller than that of poly(I) (18%). These facts suggest that the thermal stability of the multistranded complex of poly(Iz) is almost the same as the poly (I) quadruplex. Therefore, we concluded that the stability of the three(or four)-stranded complex of polymer is not affected by substitution of the hydroxyl group by the azido group at the 2'-carbon of inosinic acid in the polynucleotide chain.

Hybridization experiments of poly(Iz) with poly(C)

It is well known that poly(I) forms a double-stranded complex upon mixing with poly(C). Poly(dI) also shows similar complex formation with poly(C) or poly(dC). We examined the complex of poly(Iz) with poly(C) by the continuous variation method. Poly(Iz) and poly(C) at 0.04 mM base concentrations were mixed in various ratios as indicated

in Fig.5 in the presence of 0.15M $Na^+$ at pH 7.0. The curves at 250 nm, 260 nm and 270 nm clearly showed formation of a 1:1 complex, poly(Iz)·poly(C), as in the case of poly(I)·poly(C).

As shown in Fig. 6, this complex formation by poly(Iz) and poly(C) was also supported by measurements of CD before and after the mixing of two components. The CD curve before

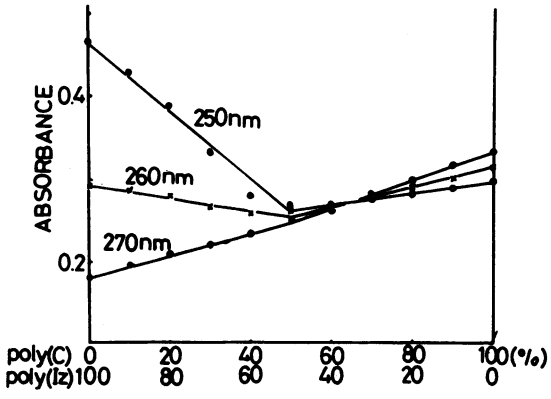



Fig.5 Mixing experiment of poly(Iz) and poly(C)

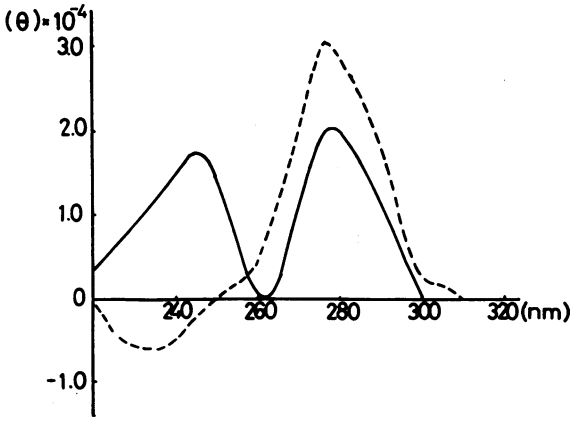



Fig. 6 CD spectra of poly(Iz) and poly(C) (1:1) before and after mixing ----- before mixing, ——— after mixing

the mixing showed a peak at 277 and a trough at 235 nm. After the mixing the curve changed to a completely different one, which had two peaks at 277 nm and 245 nm, and a trough at 262 nm. The amplitude of the long wavelength CD band descreased as compared to that before mixing, and its shorter wavelength CD band was reversed in sign with an increase in magnitude. These changes indicate complex formation in the mixture.

Thermal stability of the poly(Iz)·poly(C) complex

The temparature-absorption profile at various ionic strengths are recorded. The Tm was 41° at 0.15M $Na^+$ ion concentration, 51° at 0.15M $Na^+$ ion concentration, 58° at 0.35M $Na^+$ ion concentration, 61° at 0.55M $Na^+$ ion concentration and 64° at 0.95M $Na^+$ ion concentration. The Tm of poly (Iz)·poly(C) complex was 11° lower than that the Tm of poly(I) ·poly(C) reported to be 62.5° at 0.15M $Na^+$ ion concentration. These Tm's showed a linear relationship with the ionic concentration as shown in Fig.7.

It has become evident that the presence, absence, or modification of the 2'-hydroxyl group of polynucleotides

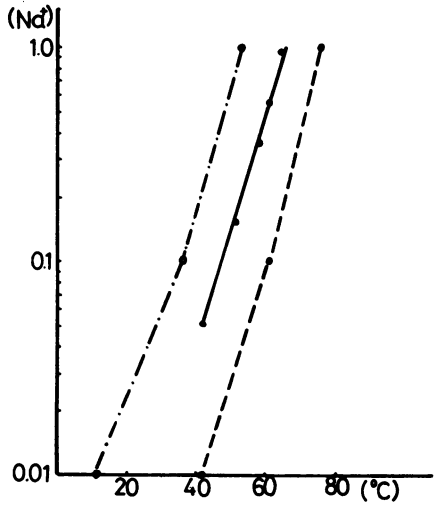



Fig.7. Relationship of $Na^+$ concentration and Tm of poly(Iz)·poly(C) ( — ), Poly(I)·poly(C)( ---- ) and poly(dI)·poly (C) ( – - – - – ).

results in significant differences in the conformation and relative stabilities of the ordered structures of such polynucleotides. Generally, double-stranded homopolymer pairs seem to follow a trend that the ribose duplexes have a higher Tm than the deoxyribose duplexes as well as the hybrid duplexes.[19] We have reported previously[10] that the poly (Az) forms a 1:2 complex with poly(U) in 0.15M $Na^+$ solution and this complex has a Tm higher than that of poly(A)·2poly(U) or poly(dA)·2poly(U).[20] In the case of the poly(Iz)·poly(C) duplex, as described above, this complex has Tm's lower than those of the ribose duplex, poly(I)·poly(C), but has Tm's higher than those of the hybrid helix, poly(dI)·poly(C), at $Na^+$ ion concentrations between 0.05 and 0.96M. These results suggest that, not only the size of the 2'-substituent, but also its interaction with solvent molecules must be taken into account for the stabilization of the complex.

In conclusion it might be emphasized that the introduction of the azido group to the 2'-position of purine nucleotides in polymer chains caused rather small changes in the physical properties as compared to ribopolynucleotides. The enchancement of interferon-inducing activity by the 2'-azido group may be ascribed to resistance of poly(Iz) to enzymatic degradation.

ACKNOWLEDGEMENT

Authors are indebted to Dr. Alexander F. Markham for reading the manuscript. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, to which our thanks are due.

REFERENCES

1. Part XLIV of this series: Morio Ikehara, W. Linn, and T. Fukui, (1977) Chem. Pharm. Bull., in press.
2. A.M. Michelson, J. Massoulie and W. Guschlbauer (1963) Progress in Nucleic Acid Res. and Mol. Biol., vol.6,p.84-141
3. J.Hobbs, H. Sternbach, M. Sprinzl and F. Eckstein (1972) Biochemistry,11,4336-4344; B. Janik, M.P. Kotick, T.H. Kreiser, L.F. Reverman, R.G. Sommer, and D.P. Wilson (1972) Biochem. Biophys. Res. Commun., 46, 1153-1160
4. P.F. Torrence, A.M. Bobst, J.A. Waters and B. Witkop (1973) Biochemistry, 12, 3962

5. J. Hobbs, H. Sterbach, M. Sprinzl and F. Eckstein (1973) Biochemistry,12,5138-5145
6. B. Zmudzka and D. Shugar (1971) Acta Biochim. Polon.,18, 321-326
7. M.K.A. Khan and F. Rottman (1972) FEBS Lett.,28, 25-28; J.T. Kusmierek, M. Kielanowska and D. Shugar (1973) Biochem. Biophys. Res. Comm.,53, 406-412
8. F. Rottman and K. Heinlein (1968) Biochemistry, 7, 2634-2641; I. Tazawa, S. Tazawa, J.L. Alderfer and P.O.P. Ts'o 1972) Biochemistry, 11, 4931-4937
9. M. Ikehara, T. Maruyama and H. Miki (1976) Tetrahedron Lett. 49, 4485-4488
10. M. Ikehara, T. Fukui and N. Kakiuchi (1977), Nucleic Acids Res., 3, 2089-2099
11. M. Ikehara, T. Fukui and N. Kakiuchi (1977), Nucleic Acids Res., in press.
12. E.De Clercq, personal communication.
13. Y. Mizuno, M. Ikehara, K.A. Watanabe and S.Suzaki (1963) J. Org. Chem., 28, 3331-3336
14. J.G.Moffatt and H.G. Khorana (1961) J. Amer. Chem. Soc., 83, 649-659
15. C.H. Fiske and Y. Subbarow (1925) J. Biol. Chem.,66, 375-400; R.J.L. Allen (1940) Biochem. J., 34, 858-865
16. F.B. Howard, J. Frazier and H.T. Miles (1971) J. Biol. Chem. 246, 7073-7086
17. A Rich (1958) Biochim.Biophys. Acta., 29, 502-509
18. S. Arnott, R. Chandrasekaran and C.M. Marttila, (1974) Biochem. J.,141, 537-543
19. M. J. Chamberlin and D. L. Patterson (1965) J. Mol. Biol., 12, 410-428
20. A. M. Michelson and C. Monny (1967) Biochim. Biophys. Acta, 149, 107-126; E. DeClercq and T. C. Merigan (1969) Nature, 222, 1148-1152