Preparation and stability of the helical form of poly 2'-O-ethyluridylic acid

J.T.Kuśmierek, Maria Kielanowska and D.Shugar

Institute of Biochemistry & Biophysics, Academy of Sciences, 02-532 Warszawa; and Dept. of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warszawa (Poland)

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Summary: 2'(3')-O-ethyl-CMP was prepared by alkylation of CMP with diethylsulphate in alkaline medium and deaminated to give 2'(3')-O-ethyl-UMP, which was phosphorylated to 2'(3')-O-ethyl-UDP. About 90% of the product consisted of the 2' isomer. The 2'(3')-O-ethyl-UDP was readily polymerized by E. coli polynucleotide phosphorylase in the presence of Mn^{++}, but not Mg^{++}. The 3'-isomer did not seriously interfere with polymerization nor did it act as a chain terminator. The resulting poly 2'-O-ethyluridylic acid formed a helical structure with a stability much higher then that of poly (rU) or poly 2'-O-methyluridylic acid. It also complexed readily with poly (rA). Implications with regard to the role of the 2'-hydroxyl in nucleic acid conformation are discussed.

No satisfactory theoretical interpretation has yet been forthcoming to account for the differences in conformational stability between the helical forms of ribo and deoxyribo polynucleotides. Proposals ascribing these differences to involvement of the 2'-hydroxyls of polyribonucleotides in some type of intramolecular hydrogen bonding are not supported by X-ray diffraction data (1,2) and are rendered doubtful, if not invalid, by the fact that poly 2'-O-methyladenylic acid (poly (Am)) (3), poly (Cm) (4) and poly (Um) (2) not only behave more like the corresponding polyribonucleotides, but exhibit higher thermal stabilities than the latter.

Attempts have consequently been directed towards the synthesis of the corresponding 2'-O-ethyl polynucleotides. One such analogue has now been described, viz. poly 2'-O-ethyladenylic acid, poly (Ae) (5), with a stability not only in excess of that for poly (rA), but also poly (Am). We report here the preparation of poly (Ue), which readily forms a helical structure with a thermal stability far surpassing that of poly (Um).

The properties of 2'-O-ethyl polynucleotides are also of interest in relation to L-ethionine induced hepatic carcinoma, which is accompanied by extensive ethylation of tRNA, an appreciable fraction of

which occurs on the 2'-hydroxyls of various residues (6).

Preparation of substrate:

To 1.1 gm (3 mM) of the sodium salt of 5'-CMP in 200 ml 0.5 N NaOH was added, stepwise, over a period of 15 hrs with constant stirring, 5 x 4 ml diethylsulphate (160 mM), each portion of DES being followed by addition of 6 ml 10 N NaOH. Chromatography of the final reaction mixture by ascending chromatography on Whatman paper No. 1, with the solvent system ethanol - 0.5 M ammonium acetate (5:2, v/v), revealed six UV-absorbing spots characterized as follows: non-reacted CMP, R_f 0.07, 35%; 2'(3')-O-ethyl-CMP, R_f 0.18, 29%; 2',3'-di-O-ethyl-CMP, R_f 0.30, 19%; and three phosphate esters with higher R_f values, 17%. Fuller details, and the mechanism of the alkylation reaction, are described elsewhere (7), but it should be noted that increasing the CMP concentration led to some alkylation of the cytosine ring N_3 and a higher yield of phosphate esters. The desired product was isolated on a preparative scale by paper chromatography, eluted with water, passed through a Dowex(H^+) column, the effluent brought to dryness under reduced pressure and the residue dried over KOH. The product could not be separated into its constituent isomers. On treatment with alkaline phosphatase it was converted quantitatively to 2'(3')-O-ethylcytidine (7); chromatography on Dowex(OH^-) according to Dekker (8), demonstrated the presence of 10-15% of the 3' isomer.

The 2'(3')-O-ethyl-CMP was deaminated with nitrite (2) and the resulting 2'(3')-O-ethyl-UMP converted according to standard procedures (9) *via* the morpholidate to 2'(3')-O-ethyl-UDP.

Preparation of poly 2'-O-ethyluridylic acid:

Although the potential substrate 2'-O-ethyl-UDP was contaminated with 10-15% of 3'-O-ethyl-UDP, previous experiments (7) with 2'(3')-O-methyl-CDP had already demonstrated that the 3' isomer did not appreciably inhibit the polymerization reaction, nor did it act as a chain terminator. Polymerization of 2'-O-ethyl-UDP was achieved with the aid of the *E. coli* enzyme, but only in the presence of Mn^{++} cations; no reaction was observed in the presence of Mg^{++} (cf.ref. 10). Following a series of trial runs, polymerization conditions employed (not necessarily optimal) were as follows: Na salt of 2'(3')-O-ethyl-UDP, 11 µM; tris buffer pH 8.5, 150 µM; $MnSO_4$, 5 µM; NaN_3, 1 µM; Na-EDTA, 0.5 µM; *E.coli* polynucleotide phosphorylase (11), 25 µl; total volume of incubation mixture, 0.75 ml. Incubation was at 37° C and the polymerization reaction was followed by TLC estimation of appearance of polymer, using Eastman 6065 cellulose plates and the solvent system isopropanol - 1% acetic acid -

2.5% ammonium oxalate (3:2:1, v/v); with this solvent system, manganese is removed from the starting point. Following 24 hours incubation, about 50% substrate incorporation into polymer was noted. The reaction was terminated by heating to 100°C, the resulting precipitate of manganese and some protein centrifuged off, and the supernatant deproteinized by the phenol procedure. The polymer solution was then dialyzed successively against 0.1 M NaCl and 0.01 M Na-EDTA, 0.01 M NaCl and 0.001 M Na-EDTA, and finally twice against water. The yield of isolated polymer was 21.5 OD_{260} units (at 18°C) which, when corrected for hypochromicity, corresponded to about 30%.

Properties of poly 2'-O-ethyluridylic acid:

Somewhat surprisingly the isolated polymer exhibited a sharp transition profile in neutral unbuffered medium, with a T_m of 22°C. This may be due to the presence of residual traces of Mn^{++} not removed by the purification procedure, and is consistent with the fact that addition of 10^{-3} - 10^{-2}M NaCl appreciably decreased the T_m, due to competition by the Na^+ ions (cf.ref. 12). At Na^+ concentrations above 0.05 M, and up to 0.8 M, the T_m increased linearly with log $[Na^+]$.

Sedimentation of the polymer in 0.1 M NaCl at 30°C, under which conditions it is in the coil form, demonstrated some heterogeneity, but with an average $S_{20} \sim 20$.

On treatment of the polymer with a mixture of snake venom phosphodiesterase, micrococal nuclease and alkaline phosphatase (10, 13), it was slowly hydrolyzed to a single product, identified chromatographically as 2'-O-ethyluridine. The extinction coeficient of the polymer in H_2O at 30°C (coil form), calculated from the phosphorus content, and with a value of 10.1×10^3 for ε_{260} of 2'-O-ethyluridine (cf.ref. 14), was 9.1×10^3. Hence the residual hyperchromicity of the coil form of poly (Ue) is 11%, comparable to that for poly (U) and poly (Um) (2).

Fig. 1(a) exhibits the transition profiles, in 0.2 M NaCl at neutral pH, for poly (U), poly (Um) and poly (Ue). The enhancement of stability of the helical form on replacement of a 2'-O-methyl by a 2'-O-ethyl is rather striking. The effect of the 2'-O-ethyl is even more pronounced in the presence of Mg^{++}, as is clear from Fig. 1(b), replacement of the 2'-O-methyl by 2'-O-ethyl leading to an increase in T_m of 22°. Profiles were fully reversible, with no observable hysteresis for the cooling profiles.

Quite remarkable was the stabilizing effect of the polyamine spermidine. On addition of one mole equivalent of spermidine to an unbuff-

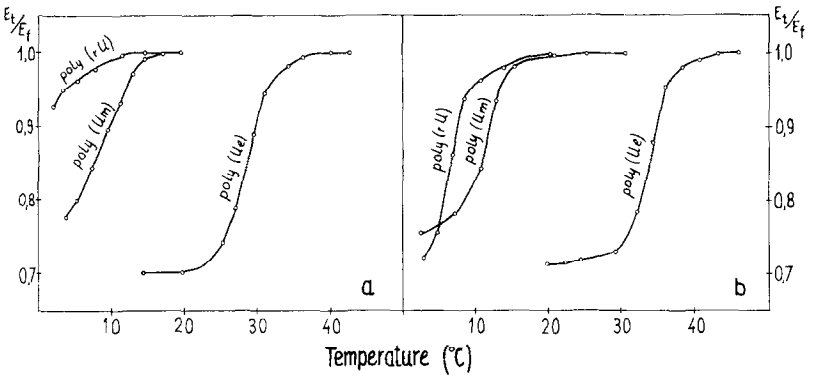

Fig. 1: Temperature transition profiles, measured at 260 nm, for poly (rU), poly (Um) and poly (Ue) in neutral aqueous medium; (a) in the presence of 0.20 M NaCl; (b) in the presence of 10^{-3} M $MgCl_2$.

ered (pH 6) solution of poly (Ue), the resulting helical structure showed no signs of melting at temperatures up to 90°C. Under analogous conditions poly (rU) melts out with a T_m of 24°C. Addition of NaCl to a final concentration of 0.01 M partially abolished the protective effect of the polyamine and gave a melting profile with a T_m of 47°, as compared to 14° for poly (rU). The unusual stabilization conferred by the polyamine and its reduction by Na^+, presumably by competition, are being subjected to more detailed investigation.

Complexing properties with poly (rA):

At neutral pH, and in the presence of 0.02 - 0.1 M Na^+, poly (Ue) readily complexed with poly (rA), revealed by hypochromicity of the mixtures. Fig. 2a exhibits the temperature profiles for 1:1 mixtures of the two components at Na^+ concentrations of 0.02, 0.04 and 0.10 M, and pointing to formation of a double-stranded helix. In order to test for possible formation of a triple-stranded helix under these conditions, mixtures of poly (Ue) and poly (rA) in the ratio 2:1 were prepared and temperature profiles run. From Fig. 2b it will be noted that, at temperatures above 40°C, the T_m values for the transition profiles at the three salt concentrations are almost identical with those for the 1:1 mixtures. The lower temperature hyperchromicity of the 2:1 mixtures may be quantitatively accounted for by the melting of the 1:1 double-stranded helix in the presence of an excess of the coil form of poly (Ue). This was further confirmed by taking advantage of the fact that, in 0.1 M Na^+, poly (Ue) itself undergoes a helix-coil transition with a T_m of 20°C; when the temperature profile of the 2:1 mixture in 0.1 M NaCl was extended to lower temperatures, a new transition profile appeared with a T_m of 20°

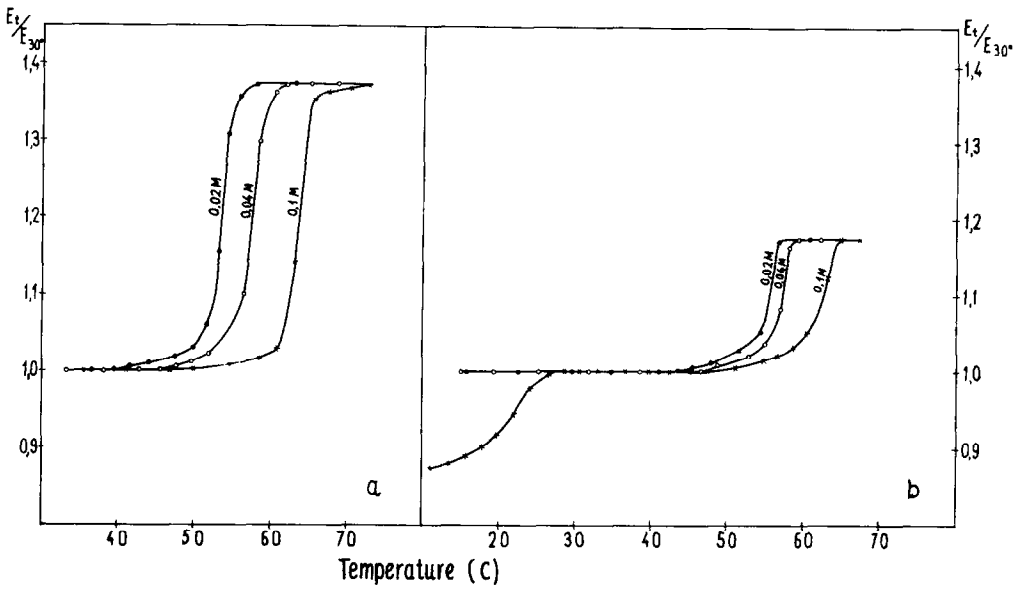

Fig. 2: Temperature transition profiles, measured at 259 nm, in 0.01 M phosphate buffer pH 7.2, and in the presence of Na^+ concentrations of 0.02, 0.04 and 0.10 M, of (a) 1:1 and (b) 2:1 mixtures of poly (Ue) and poly (rA). Note, in (b), that extension of the profile for 0.1 M Na^+ to lower temperatures places in evidence the helix-coil transition for the excess poly (Ue) in the 2:1 mixture, showing that a double-stranded complex is formed.

(Fig. 2b), corresponding to the formation of helical poly (Ue). The hypochromicity observed for this transition was quantitatively equal to that expected from the excess of free poly (Ue) in the 2:1 mixture. Hence, up to 0.1 M Na^+, poly (Ue) forms a double-stranded helix with poly (rA) irrespective of the ratio of the components. This technique should be applicable, under appropriate conditions of ionic strength, to determination of the strandedness of complexes of poly (rU) and poly (Um) by extension of the temperature range to lower values where these exhibit their own helix coil transitions (see Fig. 1a). We are currently employing this procedure to study the nature of the poly (Ue)·poly (rA) complexes at higher ionic strengths.

The T_m for double-stranded poly (rA:Ue) is virtually identical with that for double-stranded poly (rA:Um) (2) and somewhat higher than that for the 1:1 complex of poly (rU) with poly (rA). The transition profiles for the poly (rA:Ue) complex were fully reversible; but it is of interest to note that the cooling profiles exhibited appreciable hysteresis.

Discussion:

The fact that both poly (Ae) (5) and poly (Ue) exhibit further enhancement of stability of helical structures suggests that this phenomenon may be encountered with most, if not all, polynucleotides, and is supported by the fact that similar, although lower, enhancement of stability is observed on introduction of a 2'-O-methyl substituent. This effect must certainly be considered in studies on the properties of tRNA's which contain a number of 2'-O-methyl nucleosides, and of the 2'-O-ethylated residues present in the tRNA of L-ethionine induced hepatic carcinoma (6).

One of the possible sources of enhanced stability of a 2'-O-alkyl polynucleotide is a change in conformation of the pentose rings, leading to more extensive base stacking. One argument against such an interpretation is the absence of changes in conformation at the monomer level in 2'-O-methyluridine and 2'-O-methylcytidine (15,16), and in 2',3', and/or 5'-O-methylcytidine (Remin and Shugar, in preparation).

It may equally be argued that the various 2'-O-alkyl polynucleotides hitherto prepared owe their enhanced stabilities, at least in part, to the continued presence of the 2'-oxygen. This is apparently supported by the fact that poly (dU) (17), poly 2'-fluoro-2'-deoxyuridylic acid (18), poly 2'-chloro-2'-deoxyuridylic acid (19), and poly 2'-amino-2'-deoxyuridylic acid (20), all of which lack the 2'-O function are incapable of forming a helical structure. However, poly 2'-azido-2'-deoxyuridylic acid (21) does form a helical structure, the T_m for which is several degrees higher than that for poly (rU).

It is of interest to compare the effect of a 2'-O-alkyl substituent with that of a pyrimidine 5-alkyl. A 2'-O-methyl leads to enhanced helical stability, which is further enhanced by a 2'-O-ethyl. By contrast, while poly 5-methyluridylic acid forms a more stable helical structure than poly (rU), replacement of the 5-methyl by a 5-ethyl to give poly 5-ethyluridylic acid leads to a large reduction in helical stability not only with respect to poly 5-methyluridylic acid, but also poly (rU) (22).

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