Poly 2'-O-methylcytidylic acid and the role of the 2'-hydroxyl in polynucleotide structure

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Summary: In the presence of Mn^{++} cations, 2'-O-methylcytidine-5'-pyrophosphate is readily polymerized by polynucleotide phosphorylase. The neutral form of the resulting poly 2'-O-methylcytidylic acid more closely resembles the neutral, single-stranded form of poly rC than poly dC. The polymer forms a twin-stranded helix in the same pH range as poly rC, but not poly dC, showing that formation of the acid form of poly rC does not involve hydrogen bonding of the 2'-OH to a base residue or phosphate oxygen. Poly 2'-O-MeC also forms a double-stranded helix with poly rI. Implications as to the role of the 2'-OH in polynucleotide structure are briefly discussed.

The demonstration by Rottman & Henlein (1968) that 2'-O-methyladenosine-5'-pyrophosphate (2'-O-MeADP) is a substrate, albeit a poorer one than ADP, for polynucleotide phosphorylase prompted us to investigate the possibility of preparing poly 2'-O-methylcytidylic acid (poly 2'-O-MeC) with the aid of this enzyme. It may be anticipated that this polymer would be of value in studies on the nature of the differences between the acid twin-stranded forms of poly rC and poly dC, the generally accepted structures for which do not assign a specific role to the 2'-hydroxyl, the absence of which in poly dC results in formation of the so-called "acid" twin-stranded form of the latter at a pH slightly on the alkaline side of neutrality (Inman, 1964: Ts'o et al., 1966; Zmudzka et al., 1969).

More generally, a clarification of the role of the 2'-OH is of obvious importance in any interpretation of the differences in structure between RNA and DNA. There has been considerable speculation, but little concrete data, on this subject. An earlier attempt to examine the problem directly was that of Knorre & Shamovsky (1967), based on the acetylation of the 2'-OH groups in poly rU and poly rA; but the incompleteness of the reaction, and the lability and extreme bulkiness of the O-acetyl groups, are obvious hindrances in such studies. An additional advantage attaches to the preparation of the 2'-O-methyl polyribonucleotides in that 2'-O-methyl ribonucleotides themselves are widely distributed as components of bacterial

and mammalian tRNA and rRNA (Hall, 1964; Wagner et al., 1967; Isaaksson & Phillips, 1968; Lane & Tamaoki, 1969).

Synthesis of substrate

The starting material for the preparation of the potential polynucleotide phosphorylase substrate, 2'-O-MeCDP, was synthetic 2'-O- MeC (Martin et al., 1968), kindly made available by Dr. C.B. Reese. Because of the limited amount of nucleoside available, it was felt that enzymatic phosphorylation offered certain advantages. Preliminary trials showed, in fact, that 2'-O-MeC was a reasonably good substrate for wheat shoot nucleoside phosphotransferase; and, with the use of p-nitrophenylphosphate as a phosphate donor, essentially as described by Barner & Cohen (1959), it proved possible to obtain 2'-O-MeCMP in about 50% yield with simultaneous recovery of unreacted nucleoside by simple paper chromatographic separation. Synthetic methods were then employed to convert the 5'-monophosphate to the morpholidate in 90% yield, and the latter to the 5'-pyrophosphate in 74% yield, by modifications of standard procedures (Moffatt & Khorana, 1961). The 2'-O-MeCDP was further purified by ethanol precipitation from concentrated aqueous medium. These preparations, because of their more general interest, are described in detail elsewhere (Janion et al., 1969).

Preparation of poly 2'-O-MeC

Polymerization of 2'-O-MeCDP was then undertaken with preparations of polynucleotide phosphorylase from Micrococcus lysodeikticus (Matthaei et al., 1967) and Escherichia coli (Kimhi & Littauer, 1968), but with negative results, notwithstanding wide variations in the incubation conditions described by the foregoing authors. It was eventually found that replacement of the normally employed Mg^{++} cation by Mn^{++} led to slow polymerization. Fraction III of Matthaei et al. (1967) was then employed to devise an incubation medium which contained, in a total volume of 100 μl: 0.7 μM 2'-O-MeCDP; 30 μl of 0.5 M tris buffer pH 8.5; 40 μl 0.025 M $MnSO_4$; 4 μl 0.01 M Na.EDTA; 10 μl 0.01 M NaN_3; 10 μl enzyme (2.5 units, Singer & Guss, 1962). Note that, as compared to Matthaei et al. (1967), the pH has been reduced to 8.5, while the substrate concentration is 6-fold lower. The NaN_3 was included to prevent possible bacterial contamination (Rottman & Henlein, 1968) during the long incubation times required. The kinetics of the polymerization reaction were not studied, but a pronounced lag was observed in all trials. Incubation for 24 - 48 hours at 37° routinely resulted in incorporation into polymer of over 60% of the substrate, as followed by paper chromatography. The incubation mixture was then immersed in a 100° water bath for 2-3 mins, to precipitate some protein and Mn^{++}, subjected to 3

deproteinizations with phenol, and the polymer isolated by passage through a Sephadex G-50 (course) column with 0.05 M triethylammonium dicarbonate. The polymer solution was then brought to small volume, dialyzed against salt, EDTA and distilled water, and lyophilized. The overall yield, in a run with 10 mg. substrate, was 55% on a molar basis.

Properties of poly 2'-O-MeC

Sedimentation constants were obtained with a Beckman Model E instrument with ultraviolet optics on solutions in 0.05 M buffer and 0.15 M NaCl. At neutral and alkaline pH the $s_{20,w}$ values for different preparations ranged from a low of 5.9 S on one sample to between 9.0 and 14.0 S for all the others. More significant, however, was the change in sedimentation at a pH below 4.6. For the sample with an $s_{20,w}$ of 5.9 S at pH 6.5 (phosphate buffer), the corresponding value at pH 4.4 (acetate buffer) was 10.1 S. For another sample the values at pH 8 (tris buffer), 6.5 and 4.4 were, respectively, 9.0, 9.1 and 13.6 S. It consequently appears that, like poly rC, poly dC (Inman, 1964), and their 5-methyl analogues (Zmudzka et al., 1969), poly 2'-O-MeC forms an acid twin-stranded helix.

This was further supported by spectral titration, which demonstrated an abrupt transition between two forms at about pH 4.6: and by the measurement of absorption spectra and melting profiles at neutral and acid pH. The neutral (pH 6.5 and above) absorption spectrum of poly 2'-O-MeC is shown in Fig. 1 at room temperature (22°) and at 85°. The temperature profile in this range of temperatures (not shown) is quite flat and non-cooperative, as for poly rC, with a hyperchromicity at 270 mμ of 15%, as compared to about 20% for poly rC (Brimacombe & Reese, 1966), and only 1-2% for poly dC (Ts'o et al., 1966: Zmudzka et al., 1969). Furthermore, enzymatic hydrolysis with a mixture of snake venom phosphodiesterase and micrococcal nuclease (Rottman Henlein (1968) was accompanied by a hyperchromicity at 270 mμ of 37% (Janion et al., 1969) as compared to 38% for poly rC (Szer & Shugar, 1966) and 18% for poly dC (Zmudzka et al., 1969).

The absorption spectrum of the polymer at pH 4.5 (0.05 M acetate buffer, 0.1 M Na^+) is likewise typical of that for acid twin-stranded poly rC under analogous conditions (Akinrimisi et al., 1963; Langridge & Rich, 1963). The spectrum is shown in Fig. 2 for the helical twin-stranded (25°) and melted single-stranded (91°) forms. Note, in particular, the pronounced hypochromicity for the melted form at wavelengths to the red of 290 mμ, to which attention was previously drawn in the case of poly rC and poly 5MerC (Szer & Shugar, 1966). The melting profile under these conditions, exhibited in Fig. 3 for two wavelengths (270 mμ and 245 mμ) is reasonably cooperative in nature, with a T_m of about 70°, as compared to 80° for poly rC under the same con-

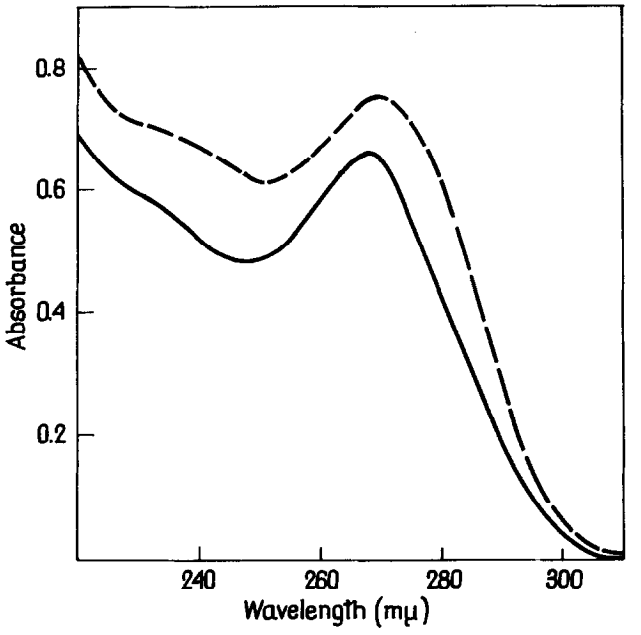

Fig. 1: Absorption spectrum of poly 2'-O-methylcytidylic acid in 0.05 M phosphate buffer, pH 6.5 and 0.1 M Na^+: ————, at 22°, and, - - - - -, at 85°.

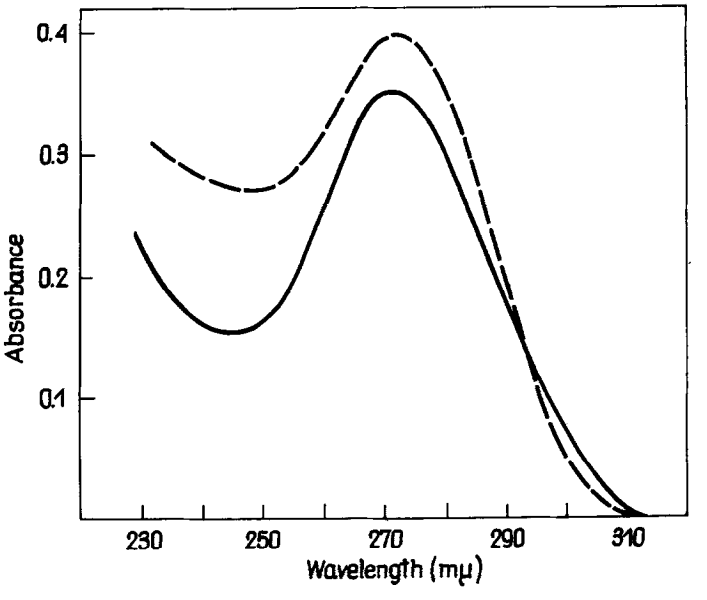

Fig. 2: Absorption spectrum of poly 2'-O-methylcytidylic acid in 0.05 M acetate buffer, pH 4.5, and 0.1 M Na^+: ————, at 25° (twin-stranded, helical form), and, - - - - -, 91° (melted form).

ditions (Akinrimisi et al., 1963). By contrast, poly dC under these conditions does not melt out at all, regardless of the Na^+ concentration (Inman, 1964; Zmudzka et al., 1969).

At neutral and slightly alkaline pH, poly 2'-O-MeC readily complexes with poly rI to form what appears to be a double-stranded helix. In 0.005 M phosphate buffer, pH 7.8, and 0.01 M Na^+, the double-stranded poly (rI: 2'-O-MeC) melts out cooperatively with a T_m of 43° and hyperchromicities of 53% and 7% at 245 mμ and 265 mμ, respectively. The T_m is therefore intermediate between that for poly (rI:rC) and poly (rI:dC), the T_m values for which, under these same conditions, are 52° and 35°, respectively. There are, however, striking differences between the absorption spectra of the helical forms of poly (rI:rC) and poly (rI:dC) (Chamberlin, 1965); and the absorption spectrum of the helical form of poly (rI:2'-O-MeC) closely resembles that for poly (rI:rC). A more extensive study of the complexes

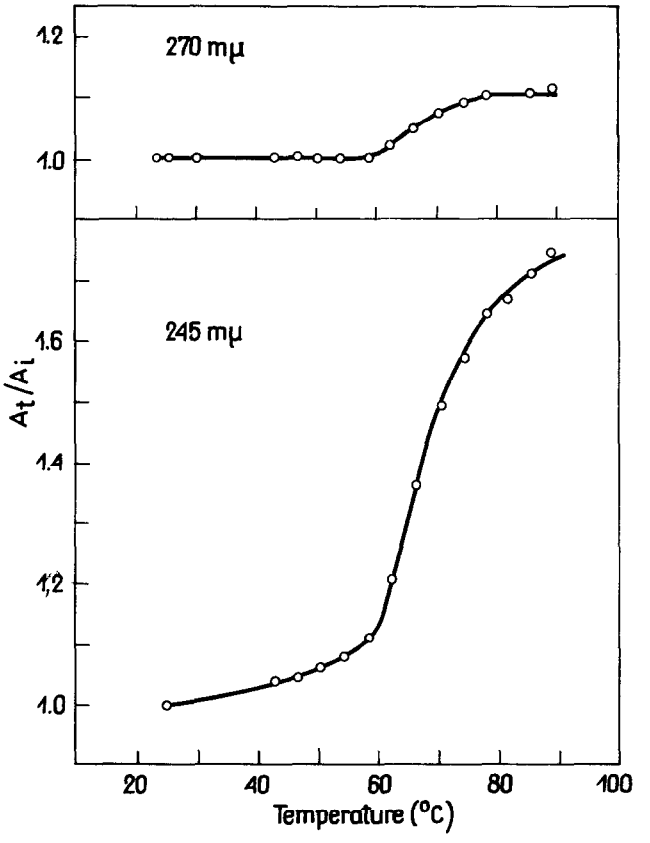

Fig. 3: Melting profiles for poly 2'-O-methylcytidylic acid in 0.05 M acetate buffer, pH 4.5, and 0.1 M Na^+, measured at wavelengths indicated. A_t/A_i is the absorbance at temperature t to that at the initial temperature (25°).

between poly 2'-O-MeC on the one hand, and poly rI and poly dI on the other, is under way.

Discussion

The apparent requirement of the M. lysod. and E. coli enzymes for Mn^{++} in place of Mg^{++}, with 2'-O-MeCDP as substrate, is quite striking. This fact is reminiscent of the observed incorporation of ribonucleoside triphosphates into a growing polydeoxyribonucleotide chain by DNA polymerase when Mg^{++} in the incubation medium is replaced by Mn^{++} (Berg et al., 1963); and prompted us to examine dTDP, which happened to be available, as a potential "substrate" for polynucleotide phosphorylase in the presence of Mn^{++} and/or Mg^{++}, but with entirely negative results (Janion et al., 1969). Nonetheless the foregoing observations clearly call for further investigation. It is perhaps worth recalling, in this connection that, while the specificity of polynucleotide phosphorylase from various sources for the base residue of the substrate is very broad, there is one rather puzzling exception, viz. 5-ethyluridine-5'-pyrophosphate is a substrate for the E. coli. but not the M. lysod. or Azotobacter vinelandii, enzymes (Swierkowski & Shugar, 1969).

The conditions for formation, and the properties, of the acid form of poly 2'-O-MeC are similar to those for poly rC, and strikingly dissimilar to those for poly dC. It logically follows that hydrogen bonding of the 2'-hydroxyl in acid poly rC, to a pyrimidine base or a phosphate oxygen, need not be invoked to interpret its difference in behaviour from poly dC. The same argument applies to the neutral form of poly 2'-O-MeC in relation to the differences between neutral poly rC and poly dC. As emphasized elsewhere (Zmudzka et al., 1969), what does still require clarification is why poly dC (and poly 5MedC) protonate to form twin-stranded helices at a pH above 7, notwithstanding that the pK_a of cytidine (and 5-methylcytidine) is close to that of deoxycytidine (and 5-methyldeoxycytidine) (Fox & Shugar, 1952). Incidentally, as might have been anticipated, the pK_a of 2'-O-methylcytidine is identical with that for cytidine (Janion et al., 1969). We shall revert to this problem and that of the acid forms of poly A in another communication.

It will be of interest to examine some of the biological properties of poly 2'-O-MeC, e.g. its behaviour as a template or as a messenger. One significant conclusion may be drawn from the ability of the polymer to complex with poly rI, viz. if poly (rI:2'-O-MeC) were to exhibit activity at least comparable to that of poly (rI:rC) in interferon induction (Field et al., 1967), it would then probably prove superior to the latter as an interferon-inducing agent, because of the complete resistance of poly 2'-O-MeC to

ribonuclease and its observed enhanced resistance to other nucleases and phosphodiesterases.

Finally, we have found that the deamination product of 2'-O-MeCDP, i.e. 2'-O-methyluridine-5'-pyrophosphate is also a substrate for polynucleotide phosphorylase; the properties of the resulting poly 2'-O-MeU will be described elsewhere.

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