SYNTHESIS OF A COPOLYMER CONTAINING ADENYLIC AND DEOXYADENYLIC ACID RESIDUES WITH POLYNUCLEOTIDE PHOSPHORYLASE

Janice Yang Chou and Maxine F. Singer

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health, Bethesda, Maryland

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SUMMARY: We report here the synthesis and characterization of copolymers containing both AMP and dAMP residues using polynucleotide phosphorylase from Micrococcus luteus. The copolymerization proceeds most readily when Mn^{2+} replaces the usual Mg^{2+} in the reaction. The dAMP residues occupy internal positions in the polymer chain, and the chains are several hundred nucleotide residues long.

Recent work has shown that polynucleotide phosphorylase from E. coli (1,2) and M. luteus[1/] can catalyze the addition of a single dAMP residue from dADP onto the 3'-hydroxyl terminus of oligoriboadenylic acids in the presence of Mg^{2+}. dADP Alone, in the presence of Mg^{2+}, is not a substrate for polymerization (1-3). On the other hand, the 2'-OH is not essential in a mononucleotide substrate since high molecular weight polymers of 2'-O-methyladenylic acid have been prepared (3). Mn^{2+} can replace Mg^{2+} in polynucleotide phosphorylase catalyzed polymerization of ribonucleoside diphosphates (4,5). Zmudka and coworkers were able to prepare poly 2'-O-methylcytidylic acid in the presence of Mn^{2+}, but not Mg^{2+} (6).

We have now found that in the absence of oligonucleotides, the polymerization of dADP occurs at a very slow rate in Mn^{2+}.[1/] Furthermore, when copolymerization of dADP and ADP is carried out in the presence of Mn^{2+}, the inhibition by dADP noted in Mg^{2+} (2)[1/] is markedly decreased permitting isolation of copolymers containing both AMP and dAMP residues in good yield. We will describe here the preparation and characterization of poly (A_5, dA).[2/]

1/ J. Y. Chou and M. F. Singer, in preparation.

2/ Abbreviations: Poly (A_5, dA) is a polynucleotide containing adenylic acid and deoxyadenylic acid in a ratio of about 5 to 1. An asterisk over either A or dA indicates ^{14}C-AMP or ^{14}C-dAMP residues, respectively.

Similar polymers with ratios of AMP to dAMP ranging from 2 to 130 have been prepared. In general the ratio of AMP to dAMP in the polymers is from 2 to 4 times the input ratio of ADP to dADP. The rate of polymerization increases with decreasing dADP. Thus far only adenine derivatives have been investigated, but there are no theoretical restrictions on preparing polymers with other bases. Detailed studies on polymerization with dADP, and the implications of those results for the mechanism of action of polynucleotide phosphorylase will be presented elsewhere.[1/]

To our knowledge there is only one additional example of enzymatic synthesis of mixed ribo-deoxyribo-polynucleotides (as distinct from end addition of, for example, ribonucleotides to polydeoxynucleotide chains). Berg and coworkers (7) reported that in the presence of Mn^{2+}, but not Mg^{2+}, E. coli DNA polymerase readily uses a ribonucleoside triphosphate in place of one of the deoxyribonucleoside triphosphates thereby producing a mixed polymer.

Materials and Methods. Nucleotides were obtained from Schwarz BioResearch or Calbiochem. Highly purified, primer-independent M. luteus polynucleotide phosphorylase was used (8). Snake venom phosphodiesterase was obtained from Calbiochem, and the Staphylococcal nuclease was a gift from Dr. P. Cuatrecasas. Bacterial alkaline phosphatase was from Worthington Biochemicals.

P_i was determined as described by Ames and Dubin (9) after removal of nucleoside diphosphates with charcoal (10). All radioactivity determinations were made in a liquid scintillation spectrometer using a triton-toluene based scintillator (11) for all aqueous samples and a toluene-based scintillator (12) for counting samples on cut-up, dried filter paper. Appropriate corrections for quenching were made.

Paper chromatography was carried out in descending fashion in the following systems: Solvent 1, 1 $\underline{M}$ NH_4HCO_3 on DEAE-paper (Whatman DE-81) (12); Solvent 2, saturated $(NH_4)_2SO_4$, isopropanol, 1 $\underline{M}$ sodium acetate (80/2/18, v/v/v) (13); Solvent 3, 95% C_2H_5OH, 1 $\underline{M}$ ammonium acetate, pH 3.8

(75/30, v/v) (14). Electrophoresis was carried out in 0.05 M potassium phosphate, pH 7.0 (15).

Preparation of Polymer. Reaction mixtures contained 0.1 M Tris·HCl, pH 9.0, 10 mM $MnCl_2$, 28 mM ADP, 20 mM dADP, and 3.5 phosphorolysis units of polynucleotide phosphorylase in a total of 0.25 ml. Two such polymers, one labeled with ^{14}C-ADP (540 cpm per nmole) and the other with ^{14}C-dADP (750 cpm per nmole) were prepared in identical fashion.[3/] After incubation at 37° for 80 min samples from each mixture were used to determine P_i release (Table I). Other samples were chromatographed in Solvent 1. Radioactivity was detected only at the origins of the chromatograms (polymer) and at the position corresponding to unused nucleoside diphosphate. The sum of the incorporation of ^{14}C-ADP and ^{14}C-dADP into polymer in the two samples was consistent with the P_i release (Table I). The remainder of each reaction mixture was passed through separate columns of Sephadex G-75 (Fig. 1A). The material eluted in the void volume of the G-75 columns, which also contains active enzyme, was used to characterize the polymer formed.

TABLE I. Synthesis of copolymer of AMP and dAMP

	Poly ($\overset{*}{A}_5$, dA)	Poly (A_5, $\overset{*}{dA}$)	Ratio AMP/dAMP	Yield of Polymer
	μmoles per 0.25 ml			μmoles
P_i released	4.4	5.0		4.7[a/]
^{14}C polymer (DEAE-paper)	3.2	0.7	4.9[b/]	3.9[c/]
^{14}C polymer (G-75)	2.9	0.5	5.8[b/]	3.4[c/]

The experiment is described in the text. The two reaction mixtures and their respective products are assumed to be identical. All results are calculated per 0.25 ml reaction mixture.

a/ Average of P_i release measured in the two mixtures.

b/ Ratio of ^{14}C-AMP incorporated into one polymer to ^{14}C-dAMP incorporated into the other.

c/ The sum of the μmoles of ^{14}C-AMP and ^{14}C-dAMP incorporated into the two polymers.

3/ The use of [8-^{3}H]-adenine nucleotides is precluded when alkaline conditions are required to characterize the polymers, because of the lability of the [8-^{3}H] at high pH (19).

Characterization of Polymer. The data in Table I show that the ratio of ADP to dADP in the product is approximately 5 to 1. The specific radioactivities of the preparations are consistent with this ratio being 415 cpm per nmole of nucleotide for poly ($\overset{*}{A}_5$, dA) and 100 cpm per nmole for poly (A_5, $\overset{*}{dA}$). The yield of polymer is approximately 30% of the input diphosphates. More than 95% of the label of both polymers was both insoluble in 5% CCl_3COOH and nondialyzable. The results of both the DEAE-paper chromatography and the G-75 columns (Fig. 1A) indicate that no oligonucleotide material is present in the polymers. These procedures together would detect material of chain length 40 and below. Fig. 1B shows sucrose gradient analysis of the two preparations. The two preparations are, as expected, similar, with the peak of material at approximately 7 S, suggesting an average chain length of about 200 or more residues for most of the polymer (16). This was confirmed by electrophoresis in 5% polyacrylamide gels at pH 7 (17) and in gels containing sodium dodecylsulfate (18).

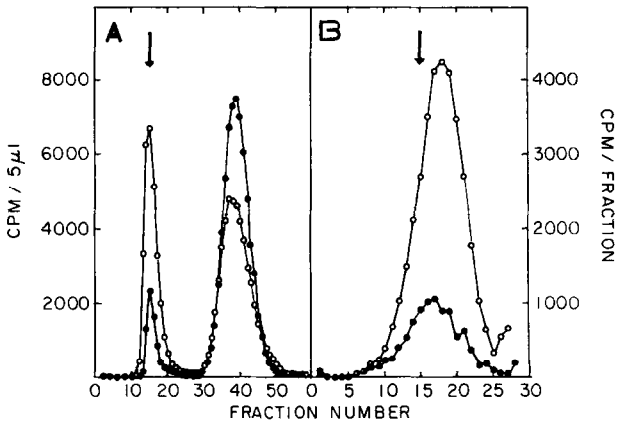

Figure 1A. Gel filtration of polymers on Sephadex G-75. Approximately 0.23 ml of the reaction mixtures described in the text was passed, at room temperature, through separate columns (0.9 x 10 cm) equilibrated with 0.01 M Tris·HCl, pH 8.2, 0.1 M NaCl, 5 mM $MgCl_2$. Fractions (about 0.2 ml) were collected and samples analyzed for radioactivity and polynucleotide phosphorylase. -O-, poly ($\overset{*}{A}_5$, dA); -●-, poly (A_5, $\overset{*}{dA}$). Arrow shows position of enzyme.
Figure 1B. Sucrose Density Gradient centrifugation of polymers. Samples of polymers isolated from G-75 columns (Fig. 1A) were layered on separate 5-20% sucrose gradients in 0.01 M Tris·HCl, pH 8.2, 1 mM $MgCl_2$, 0.2 M NaCl and spun for 12 hr at 35,000 rpm in an SW-39 rotor. After collection of the gradient fluid, samples were analyzed for radioactivity and polynucleotide phosphorylase ($S_{20,w} \simeq 9.0$ (8)). Sedimentation was from right to left. Symbols as for Fig. 1A.

TABLE II. Radioactive products of copolymer degradation.

Treatment	Poly ($\overset{*}{A}_5$, dA)		Poly (A_5, $\overset{*}{dA}$)	
	Product	Percent[a]	Product	Percent[a]
Venom phosphodiesterase[b]	5'-AMP	100	5'-dAMP	100
Staphylococcal Nuclease[c]	3'-AMP	99	3'-dAMP	98
KOH[d]	dAprAp	11	dAprAp	88
	2',3'-AMP	85		

a/ The percent of the total counts recovered from paper strips found in the compound named. The remaining radioactivity was in compounds representing polymer end groups. Essentially all the radioactivity applied to the chromatograms was recovered in each case.

b/ Digests chromatographed in Solvent 1. Under the conditions used 3 to 25% of the polymer remained undegraded (at the origin). In each case only a single degradation product was observed; that is, 100% of material migrating away from origin was AMP or dAMP.

c/ Digests were applied to paper for electrophoresis. Degradation was complete. Upon elution and rechromatography in Solvent 2, 92% of the product from poly ($\overset{*}{A}_5$, dA) was found at the mobility expected for 3'-AMP.

d/ Digests were carried out in 0.5 M KOH for 18 hr at 37°, and neutralized to pH 8 with Dowex-50, H^+. Some digests were chromatographed in Solvent 3, which separates dinucleotides ($R_{3'\text{-AMP}}$=0.54) from AMP. Others were chromatographed in Solvent 2 ($R_{3'\text{-AMP}}$ of pApA=0.60).

These results show that both the dADP and ADP are present in long polymer chains. That copolymerization did indeed occur was proven by specific degradation of the polymers. Table II outlines the major degradation products of the copolymer after treatment with Staphylococcal nuclease, snake venom phosphodiesterase, or alkali. The minor products expected from the ends of the polymers have been ignored. The radioactive products actually found (Table II) are those expected for a copolymer of AMP and dAMP residues in which the dAMP occupies internal positions in the chains. Snake venom diesterase yielded ^{14}C-5'-AMP and ^{14}C-5'-dAMP and Staphylococcal nuclease yielded ^{14}C-3'-AMP and ^{14}C-3'-dAMP from poly ($\overset{*}{A}_5$, dA) and poly (A_5, $\overset{*}{dA}$), respectively. In the latter case, the product from poly (A_5, $\overset{*}{dA}$) would have been ^{14}C-deoxyadenosine if all the dAMP residues had been at the 3'-hydroxyl terminus. The results with alkali confirm this: 85% of the label from alkali-degraded

poly ($\overset{*}{A}_5$, dA) was in 2'-3'-AMP and 12% in a dinucleotide, while most of the label from poly (A_5, $d\overset{*}{A}$) was in the dinucleotide. Again, if all the dAMP residues had been at the 3'-terminus of poly (A_5, $d\overset{*}{A}$) alkaline digestion would have yielded ^{14}C-deoxyadenosine. It is not known whether the distribution of dAMP residues within the chains is random.

The dinucleotide product from alkaline hydrolysates was identified as dAprAp in the following manner. Treatment with bacterial alkaline phosphatase converted the radioactivity of dinucleotide labeled either in dAMP or rAMP to material with the electrophoretic mobility of ApA. The original material was relatively resistant to venom phosphodiesterase, as expected, but in one experiment with dAp$\overset{*}{r}$Ap 30% of the counts were recovered as ^{14}C-pAp. If the dinucleotide was treated with venom diesterase in the presence of alkaline phosphatase the labeled product was deoxyadenosine or adenosine from $d\overset{*}{A}$prAp or dAp$\overset{*}{r}$Ap, respectively. Treatment with Staphylococcal nuclease yielded ^{14}C-3'-dAMP or ^{14}C-3'-AMP, depending on the original label.

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