DEOXYADENOSINE DIPHOSPHATE AS SUBSTRATE FOR POLYNUCLEOTIDE PHOSPHORYLASE FROM *ESCHERICHIA COLI*

G.KAUFMANN and U.Z.LITTAUER
Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Until now deoxynucleoside diphosphates were not considered as substrates for the enzyme polynucleotide phosphorylase. This belief was based on the following observations: (a) The enzyme catalyzes the exchange reaction between orthophosphate and the β-phosphate by dTDP at a rate which is about 100-fold slower than the rate of P_i exchange with ADP [1]. (b) The enzyme cannot perform *de novo* polymerization of dADP [2]. (c) DNA is not phosphorolyzed by the enzyme [3].

We have recently observed that polynucleotide phosphorylase can phosphorolyze aminoacyl-tRNAs [4] yielding thereby aminoacyl-ADP and nucleoside diphosphates. This finding prompted us to investigate the properties of ribonucleoside diphosphate analogs modified in their sugar moiety as substrates for this enzyme. In this communication we show that 2′-dADP can participate in the nucleoside diphosphate-P_i exchange reaction as well as in a limited and reversible polymerization in the presence of oligoribonucleotide initiators.

2. Experimental

dADP was purchased from Sigma and (^{14}C)dADP was obtained from Schwarz Bioresearch, Inc. *E. coli* polynucleotide phosphorylase was purified and assayed as previously described [5]. The specific activity of the enzyme was 100 units/mg protein. ApA and ApU were obtained from Zellstofffabrik, Waldhof.

The following solvent systems were used for paper chromatography: (A) Isobutyric acid, 1 N NH_4OH, 0.1 M EDTA; 50:30:08; (B) Ethanol, 1 M Ammonium acetate, pH 7.0; 40:60 to 50:50 as indicated; (C) Ethanol, 1 M Ammonium acetate, pH 3.8; 7.5:3. Whatman No. 1 filter paper was used throughout the experiments. For the measurement of radioactivity the chromatograms were cut into narrow strips and counted with 10 ml toluene scintillation fluid. Liquid samples from column fractions were counted with 10 ml dioxane scintillation solution [6]. Total phosphate determination was carried out according to Ames and Dubin [7].

3. Results

3.1. dADP-$^{32}P_i$ *exchange catalyzed by polynucleotide phosphorylase*

Polynucleotide phosphorylase normally catalyzes an exchange reaction between the β-phosphate of a ribonucleoside diphosphate and inorganic phosphate. When 2′-dADP was incubated with polynucleotide phosphorylase and $^{32}P_i$ under the standard nucleoside diphosphate-P_i exchange conditions, $^{32}P_i$ was incorporated into dADP. The rate of this reaction was about 200-fold slower than that of ADP-$^{32}P_i$ exchange. It seemed that the observed exchange was not due to ribonucleoside diphosphate contaminants in the dADP or in the enzyme preparation since on prolonged incubation the specific radioactivity of dADP equilibrated with that of $^{32}P_i$. When the reaction products were separated by paper chromatography the incorporated $^{32}P_i$ was found exclusively in dADP, as shown in fig. 1. In this solvent system dADP migrates ahead of the 4 common ribonucleoside diphosphates.

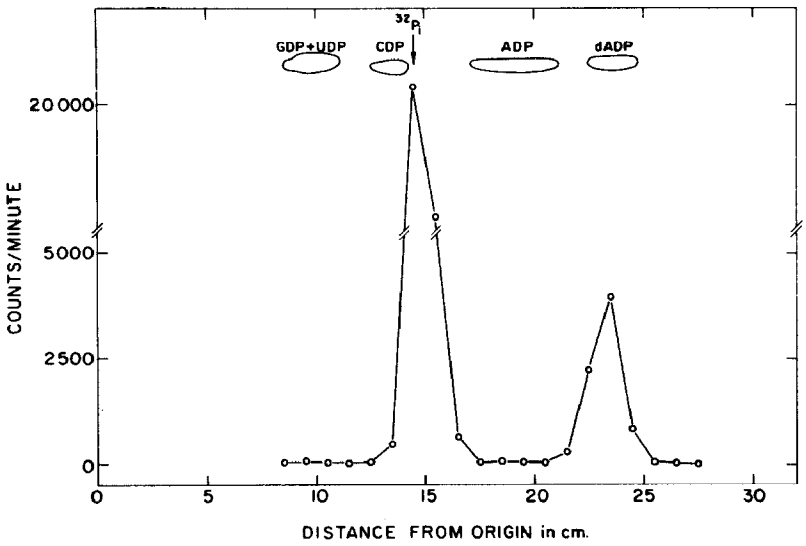

Fig. 1. Chromatographic separation of dADP-$^{32}P_i$ exchange products. The reaction mixture (0.25 ml) contained: 0.8 mM dADP, 2 mM (^{32}P) potassium phosphate (2.10^6 cpm/μmole) pH 8.0, 4 mM $MgCl_2$, 0.2 M Tris-HCl pH 8.3 and 0.7 units of polynucleotide phosphorylase. The incubation was carried at 37° for 20 hr at the end of which 0.1 ml of 10% perchloric acid was added. The mixture was centrifuged and 10 μl of the supernatant were chromatographed with solvent system A for 18 hr.

3.2. *Absence of de novo polymerization of dADP*

dADP was incubated with an excess of enzyme for 72 hr. The reaction mixture (0.05 ml) contained: 30 mM (^{14}C) dADP (0.5 μc/μmole); 5 mM $MgCl_2$; 0.1 M Tris-HCl buffer pH 8.2 and 0.2 units of purified polynucleotide phosphorylase. 10 μl aliquots were withdrawn and heated for 2 min at 100°, chilled and incubated for 3 hr at 37° with 0.1 units of *E. coli* alkaline phosphatase in order to convert the dADP to deoxyadenosine. The products were separated by paper chromatography with solvent system B (50:50). The accuracy of the method allowed us to detect dinucleotides or higher oligonucleotides in amounts less than 0.1% of the input dADP. However, only dA was found. This result is in accord with the data presented recently by Rottman and Heinlein [2].

3.3. *Limited polymerization of* dADP *by polynucleotide phosphorylase in presence of short oligoribonucleotide initiators*

The inability of polynucleotide phosphorylase to perform *de novo* polymerization of dADP could be due to either the failure of the enzyme to transfer deoxyadenylyl residues or to the inability of deoxynucleotides to serve as acceptors for the incoming nucleotidyl residue. The second possibility seemed more likely since, as shown above, polynucleotide phosphorylase catalyzed the dADP-$^{32}P_i$ exchange reaction. It was therefore expected that the enzyme would carry out some polymerization of dADP in the presence of an oligoribonucleotide with a terminal free 3′-hydroxyl group which would serve as an initiator for the reaction.

In order to test this assumption (^{14}C)dADP was incubated with large amounts of polynucleotide phosphorylase in presence of ApA. The molar ratio of dADP: ApA was 2:1. Aliquots from the reaction mixture were separated by paper chromatography with solvent B (50:50). In addition to ApA and dADP two slower migrating ultra-violet absorbing spots appeared after prolonged incubation. These two new spots were found to be radioactive. From the ratio of cpm/O.D. and from the position on the chromatogram it seemed possible that these spots were the trinucleotide ApAp (^{14}C)dA and the tetranucleotide ApAp (^{14}C)dAp. (^{14}C)dA which had arisen by the respective addition of one and two (^{14}C)deoxyadenylyl residues to ApA.

In order to confirm the proposed structure of the new oligonucleotides a large-scale reaction was set up. After phosphatase treatment the products of the

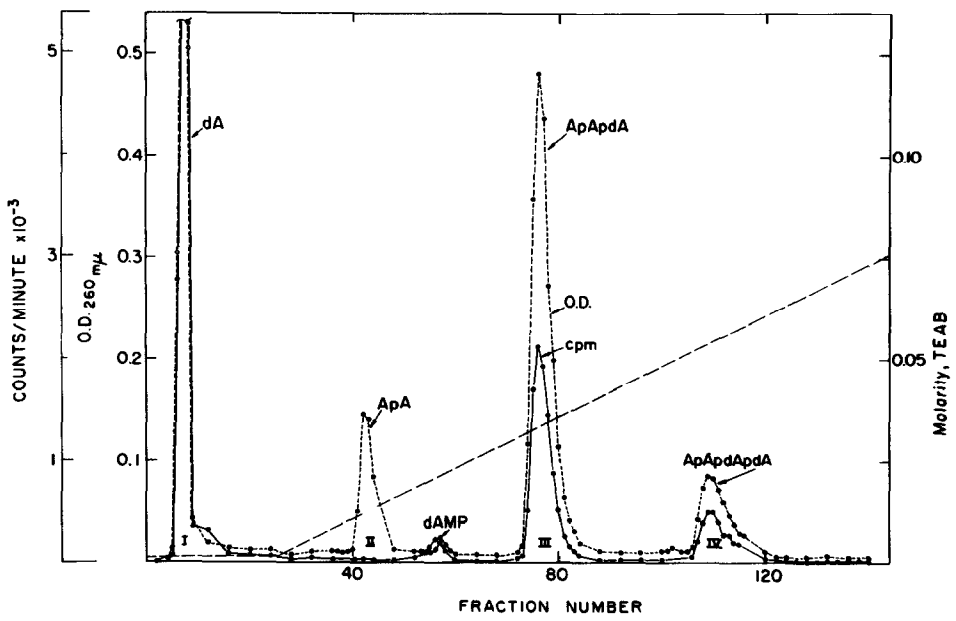

Fig. 2. Separation of the products of dADP polymerization in the presence of ApA on DEAE-cellulose column chromatography. The reaction mixture (0.5 ml) contained: 10 mM (^{14}C)dADP (0.25 μC/μmole), 5 mM ApA, 5 mM $MgCl_2$, 0.1 M Tris-HCl pH 8.2 and 1.6 units of polynucleotide phosphorylase. Incubation was carried out at 37° for 48 hr at the end of which the reaction mixture was heated for 2 min at 100° and chilled. 0.4 units of *E. coli* alkaline phosphatase were added and incubation was carried out in a final volume of 1.5 ml for 3 hr at 37°. The incubation mixture was then heated for 2 min at 100°, diluted with 75 ml 0.005 M TEAB (triethylammonium bicarbonate) and applied to 20 X 2 cm DEAE-cellulose (1 meq/g) column equilibrated with the same buffer. The column was washed with 500 ml of 0.005 M TEAB and eluted with a linear gradient consisting of 2 liters each of 0.005 M and 0.5 M TEAB. Fractions of 20 ml were collected.

reaction were separated on a DEAE-cellulose column. Fig. 2 shows the elution pattern of the reaction mixture. Peak 1 corresponds to (^{14}C)deoxyadenosine which resulted from the unreacted (^{14}C)dADP, peak 2 is the unreacted ApA while peaks 3 and 4 are the new products of the reaction. The tubes containing peaks 3 and 4 were pooled, concentrated by lyophylization and analyzed by the following methods: Total phosphate determination, complete alkaline hydrolysis and complete digestion with snake venom phosphodiesterase. Table 1 summarizes the properties of compounds 3 and 4. The ratio of (^{14}C)deoxyadenylyl residues to phosphate and to the total nucleotide residues as well as the products of phosphodiesterase and alkaline digestions clearly demonstrate that peak 3 is ApAp(^{14}C)dA and peak 4 is ApAp(^{14}C)dAp(^{14}C)dA. It should be noted that the venom phosphodiesterase contained traces of phosphatase therefore the values for dAMP are slightly lower than the theoretical.

In the same manner ApU was tested as an initiator for (^{14}C)dADP polymerization in the presence of polynucleotide phosphorylase. The separation of the products was carried out by DEAE-cellulose column chromatography. In addition to the unreacted monomer and ApU two new oligonucleotides were detected. The elution pattern and the ratio of radioactivity to O.D. units suggested that these compounds were ApUpdA and ApUpdApdA. The first substance was subjected to the same analyses which served for the proof of the structure of ApApdA. Table 1 summarizes these results from which it is evident that the material tested was indeed ApUpdA. The amount of the second peak was too low to enable further analysis.

3.4. *Phosphorolysis of ApApdA*

DNA is not phosphorolyzed by polynucleotide phosphorylase [3]. Yet it seemed possible that the enzyme might catalyze the reverse reaction of ApApdA

Table 1
Products of dADP polymerization in presence of ApA or ApU.

Oligonucleotide initiator	Peak	$a_{260} \times 10^{-3}$ at pH 2	Total phosphate[a] (moles/chain)	(^{14}C)deoxyadenosine[b] residues (moles/chain)	Products of[c] alkaline hydrolysis	Products of[d] digestion with venom phosphodiesterase	Products of[e] phosphorolysis	Structure derived
ApA	3	40.0	2.0	1.0	2Ap,dA 2.4, 1.0	A,pA,pdA 1.3, 1.3, 1.0	ApA,ppdA	ApApdA
ApA	4	53.4	2.5	2.0	2Ap,dApdA[f] 2.2, 1.0	A,pA,2pdA 1.1, 1.2, 2.0	-- --	ApApdApdA
ApU	3	40.5	1.7	1.0	Ap,Up,dA 1.1, 1.1, 1.0	A,pU,pdA 1.3, 1.2, 1.0	-- --	ApUpdA

The oligonucleotides were synthesized and isolated as described in the legend to fig. 2. The values are expressed as moles/chain using the (^{14}C) deoxyadenosine residues as the standard. a. Total phosphate was determined on 0.5–1.0 A_{260} units of oligonucleotide. b. The specific radioactivity of (^{14}C) deoxyadenosine residues was 156,000 cpm/μmole. c. Alkaline hydrolysis was carried out in 0.3 N KOH at 37° for 16 hr. The hydrolysate of ApApdA was separated by paper chromatography with solvent C and those of ApUpdA and ApApdApdA were separated by paper electrophoresis at pH 1.9. d. The digestion mixture (0.02 ml) contained 2–3 A_{260} units of oligonucleotide, 0.02 M Tris HCl pH 8.9, 0.01 M $MgCl_2$ and 4 μg of snake venom phosphodiesterase (Worthington). The incubation was carried out for 3 hr at 37°. The digest was separated by paper chromatography with solvent system A. e. The products of ApApdA phosphorolysis were identified as described in the legend to fig. 3. f. The structure of dApdA was proven by snake venom phosphodiesterase treatment which gave rise to dA and dAMP.

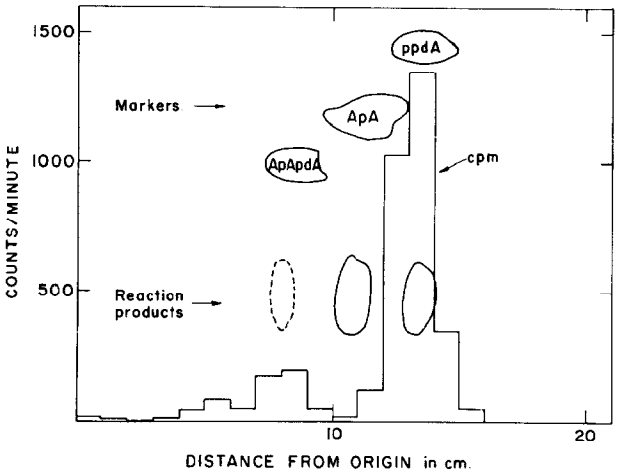

Fig. 3. Separation of ApAp(^{14}C)dA phosphorolytic digest by paper chromatography. The reaction mixture (0.1 ml) contained: ApAp(^{14}C)dA 3.4 A_{260} units (3980 cpm/A_{260} unit), 5 mM $MgCl_2$, 10 mM potassium phosphate pH 8.0, 100 mM Tris-HCl pH 8.0 and 0.7 units of polynucleotide phosphorylase. The incubation was carried out at 37°. After 24 hr 20 μl of the reaction mixture were chromatographed on paper with solvent system B (40:60).

synthesis. Fig. 3 shows that incubation of ApAp(^{14}C) dA with polynucleotide phosphorylase and phosphate yielded ApA and (^{14}C)dADP.

4. Discussion

2′-dADP differs from the normal substrates of polynucleotide phosphorylase in some respects. The enzyme catalyzes a slow dADP-$^{32}P_i$ exchange reaction but does not polymerize dADP *de novo*. In the presence of oligoribonucleotides such as ApA or ApU there occurred a limited polymerization of dADP. Under the conditions employed one or two deoxyadenylyl residues were added to the oligonucleotide initiator. The normal substrate yields under the same conditions highly polymerized products only [8]. In the limited polymerization of dADP one DNA-like internucleotide linkage was formed thus showing that the 3′-hydroxyl of dADP has not altogether lost its acceptor function. The reason why the polymerization of dADP could not proceed readily beyond the tetra-

nucleotide stage seems to be due to the low affinity the enzyme to the DNA-like internucleotide linkage. One can visualize a binding site in the enzyme to which the 3′-terminus of the growing polynucleotide is attached. This presumed site recognizes a portion of the ribopolynucleotide backbone several phosphodiester bonds in length. When deoxyadenylyl residues are added to the growing end of the chain its affinity to the enzyme is lowered and the rate for further elongation is hence greatly reduced.

Two mechanisms were suggested for the ribonucleoside diphosphate-P_i exchange reaction: (a) The observed exchange reflects a reversible formation of a nucleoside monophosphate enzyme complex or (b) the apparent exchange is a result of combined polymerization and phosphorolysis reactions occurring under approximate equilibrium conditions [3]. The existence of a substrate such as dADP which participates in the exchange reaction but is not polymerized *de novo* supports the first hypothesis. The properties of dADP may also facilitate the isolation of the presumed enzyme-substrate complex since there is no interfering polymer formation. However, at present one cannot rule out the possibility that the apparent dADP-P_i exchange is due to the presence of trace amounts of oligoribonucleotides in the enzyme preparation.

These assumptions as well as other properties of deoxynucleoside diphosphate substrates of polynucleotide phosphorylase are the subject of further studies.

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