Enzymatic synthesis of deoxyribo-oligonucleotides of defined sequence.
Properties of the enzyme*

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Received 30 May 1974

ABSTRACT

A modified purification is described for an enzyme, from *Escherichia coli* B, which polymerizes deoxyribonucleoside-5' diphosphates. Under appropriate conditions, the enzyme will add a single deoxyribonucleotide residue to a deoxyribo-oligonucleotide primer. At all stages, the enzyme activity copurified with the activity which will polymerize adenosine-5' diphosphate (polynucleotide phosphorylase). Studies of heat stability, the effect of various temperatures of reaction and of disc gel electrophoresis failed to provide evidence that the two activities are separable.

INTRODUCTION

We have recently reported the use of an enzyme preparation from *E. coli* B to add a single deoxyribonucleotide derived from a deoxyribonucleoside-5' diphosphate to the 3'-terminus of an oligodeoxyribonucleotide (1). The objective of these experiments is a convenient method for the stepwise synthesis of oligodeoxyribonucleotides of defined sequence. The present paper describes the properties of the enzyme which was originally discovered by Hsieh (2,3,4) as an activity which polymerizes deoxyribonucleoside-5' diphosphates. Comparison indicates that the enzyme is probably identical with *E. coli* polynucleotide phosphorylase (5). Similar conditions to those previously applied to polynucleotide phosphorylase to achieve oligoribonucleotide synthesis (6) have been successfully applied to the synthesis of oligodeoxyribonucleotides, and the basic procedures are described. A following paper will describe the application of the method to the preparation of a variety of oligodeoxyribonucleotides (7).

METHODS

Materials - Unlabeled deoxyribonucleoside diphosphates, snake venom phosphodiesterase and *E. coli* alkaline phosphatase were obtained from P-L Biochemicals; [^3H]dADP, from Schwarz BioResearch; [^3H]ADP from New England Nuclear; Sephadex G-200, and Sephadex G-100, from Pharmacia Chemical and DEAE-cellulose was Whatman DE-22. NCS solubilizing agent used in liquid scintillation counting was purchased from Nuclear Chicago. Aquasol and Omnifluor are products of New England Nuclear. *E. coli* B cells were purchased from Grain Processing Corp. Deoxyoligonucleotide primers were synthesized chemically (8). The enzyme was isolated as described by Hsieh (2) with the addition of two Sephadex gel filtration steps. The detailed procedure for Sephadex G-200 filtration is given in Fig. 1. Fractions containing the enzyme activity were pooled and concentrated by ammonium sulfate precipitation at 50% saturation. The precipitate was dissolved in the same eluting buffer as for Sephadex G-200 column (27 mg in 2 ml) and applied to a Sephadex G-100 column (1.5 x 45 cm).. Fractions at 1.0 ml were collected at 15 minute intervals. All the studies reported here were carried out with the enzyme fraction after elution from Sephadex G-100.

Assays - The standard assay for enzyme activity was determined by the incorporation of [^3H]dADP into acid-insoluble polymers. The incubation mixture (0.2ml) contained 0.1 M Tris-HCl pH 8.5, 0.01 M 2-mercaptoethanol, 0.01 M MnCl_2 , and 1.25 mM [^3H]dADP (2×10^6 cpm/ μmole). The reaction was initiated by addition of the enzyme and was incubated at 37°C for 20 min. The reaction was terminated by the addition of 3 ml of 5% TCA. The precipitate was collected by centrifugation and washed three times with 3 ml of 5% TCA. 1 mg bovine serum albumin in each tube was used as carrier during the TCA washing steps. The washed precipitate was solubilized in 0.3 ml NCS and transferred to a scintillation solution. The radioactivity was determined in a scintillation counter at an efficiency of 30% for ^3H and 80% for ^{14}C . For the determination of ADP polymerization activity, dADP was replaced by ADP and MnCl_2 by MgCl_2 . The protein concentration was determined either by a biuret method or

spectrophotometrically by measuring the absorption at 215 nm and 225 nm (10). One unit of enzyme was defined as the amount catalyzing the conversion of 1 nmole of [^3H]dADP (or [^3H]ADP) into acid-insoluble material in one minute.

Gel electrophoresis - Polyacrylamide gel electrophoresis was carried out according to Ornstein (11) and Davis (12). Enzymatic activity was localized by the use of a gel slicer. Two gel slices (1 mm per slice) were homogenized in 0.3 ml Buffer (Tris-HCl), 0.1 M, pH 8.5 and 0.01 M 2-mercaptoethanol. After two hours extraction at 0°, the gel was removed by centrifugation and 50 μl of the eluate was used for assay of ADP polymerization and 150 μl for dADP polymerization activity. To localize the protein bands, the gels were stained overnight with 0.25% Coomassie Brilliant Blue in 7.5% acetic acid, and destained with 7.5% acetic acid.

Deoxyoligonucleotide synthesis - The reaction mixture (1 ml) contained primer; radioactive deoxyribonucleoside-5'-diphosphate 1.5 μmoles ; MnCl_2 , 10 μmoles ; NaCl, 300 μmoles ; 2 mercaptoethanol, 15 μmoles ; Tris-HCl (pH 8.5), 100 μmoles and enzyme, 1.8 mg. Reaction mixtures were incubated at 37° for 4 hours and stopped by addition of 0.4 ml of 0.1M EDTA, pH 7.0. The reaction mixture was analyzed on a DEAE-cellulose column (1.2 x 10 cm) in the carbonate form. The column was eluted at 25° using a linear gradient of ammonium bicarbonate. The products, $\text{pd}(\text{A}_5)$ and $\text{pd}(\text{A}_6)$ were first dephosphorylated with *E. coli* alkaline phosphatase and the oligonucleotides were freed of phosphomonoesterase by paper chromatography in solvent 2. The nucleotide compositions of the dephosphorylated oligonucleotide were determined by degradation of each oligonucleotide with snake venom phosphodiesterase at 37° for 3 hours and separation of the nucleotides by paper chromatography. Paper chromatography was carried out in descending fashion in the following systems:

solvent 1: saturated $(\text{NH}_4)_2\text{SO}_4$ / 0.6M sodium acetate/
2-propanol (160/36/4 v/v/v).

solvent 2: 1-propanol/ NH_4OH / H_2O (55/10/35 v/v/v)

solvent 3: Isobutyric acid /1M NH_4OH (5/3, v/v, pH 4.4)

RESULTS

Specificity of the enzyme. Polynucleotide phosphorylase from *E. coli* can catalyze the addition of a single dAMP residue from dADP onto the 3'-OH terminus of oligoriboadenylic acids in the presence of Mg^{++} (13,14). Polynucleotide phosphorylase from *Micrococcus luteus* can readily catalyze the formation of copolymers containing AMP and dAMP residues when Mn^{++} replaces Mg^{++} in the reaction (15). Since the *E. coli* enzyme, with the appropriate cation catalyzes the polymerization of ADP at a much faster rate than that of dADP (1,2), it was of interest to examine whether the various enzyme fractions obtained during the different steps of purification would show the same relative degree of purification when assayed by the polymerization of dADP and ADP. Comparison of the two activities during the process of purification showed that they are associated throughout (Table 1, Fig. 1). The difference in the ratio of activities at different stages could be due to the presence of ribonucleases in the crude homogenate and early fractions. Thang et al. (16) have shown that in *E. coli* B and K_{12} , there is small quantity of a low molecular weight polynucleotide phosphorylase which catalyzes only the phosphorolysis of polyribonucleotides. The possibility of the catalysis of dADP polymerization by a low molecular weight enzyme was examined by determining polymerization activities for dADP and ADP after chromatography of the enzyme on Sephadex G-200 and Sepharose 6B columns. The elution profile and enzymatic activities from the Sepharose 6B columns is shown in Fig. 2. It is evident that there was no separation of the two activities.

Thermal Stability of the enzyme with respect to dADP and ADP polymerization reactions: It is known that polynucleotide phosphorylase from *E. coli* is stable up to 55° and that poly A protects the enzyme against thermal inactivation (17). If the polymerization of dADP and ADP reactions are carried out by separate enzymes the stability can be used as a tool to distinguish between the two. The results of experiments along these lines are given in Fig. 3. It is clear that under these conditions, both polymerization activities followed the same

TABLE I

Comparison of dADP and ADP polymerization activities
at different stages of enzyme purification.

Fractions	dADP polymerization activity		ADP polymerization activity	
	specific activity units/mg protein	total activity units	specific act. units/mg pro.	total act. units
Crude extract	0.056	375	0.24	1,550
Streptomycin sulphate	0.098	440	0.784	3,500
Acid precipita- tion	0.11	360	1.03	3,300
(NH ₄) ₂ SO ₄	0.53	430	11.7	9,400
DEAE-cellulose	0.985	370	19.7	7,400
Phosphocellulose	3.68	220	103	6,100
Sephadex G-200	4.46	120	130	3,400
Sephadex G-100	4.52	90	125	2,440

100 g of frozen *E. coli* B cells were used for the preparation of the enzyme. The enzyme was purified according to Hsieh (2) with the addition of the Sephadex chromatography steps.

Fig. 1. Chromatography on Sephadex G-200

Protein from the phosphocellulose step (58 mg in 5 ml) was applied to a Sephadex G-200 column (1.5 x 90 cm) which had been equilibrated with the eluting buffer (0.05 M, Tris-HCl, pH 7.8; 0.01 M, 2-mercaptoethanol; 0.05 M, NaCl; 5% glycerol). Fractions were collected at 30 minute intervals at a rate of 1 ml/10 minutes.

Fig. 2. Gel filtration on Sepharose 6B.

The enzyme from the Sephadex G-100 fraction (7 mg in 1 ml) was applied to a Sepharose 6B column (1.2 x 60 cm) which had been equilibrated with eluting buffer (0.05 M; Tris-HCl, pH 7.8; 0.01M 2-mercaptoethanol; 0.05 M, NaCl; 5% glycerol). The column was eluted with this buffer at a flow rate of 1 ml/30 minutes. Fractions were collected at 20 minute intervals.

Fig. 3. Heat stability of the enzyme.

50 μ g of enzyme was preincubated at different temperatures for 10 minutes in 0.15 ml of a mixture containing 0.12 M Tris-HCl, pH 8.5, 0.01 M 2-mercaptoethanol, alone or with nucleoside diphosphate or poly A. The mixture was then cooled to 0° and the components necessary for the reaction were added. The resulting mixtures were incubated at 37° for 20 minutes for the dADP polymerization (B) and 10 minutes for the ADP polymerization reaction (A). Activity in buffer alone (▲---▲---▲); in the presence of nucleoside diphosphate 0.65×10^{-3} M (●---●---●); in the presence of poly A, 50 μ g (□---□---□).

patterns with a precipitous fall in the enzymatic activity to nearly complete inactivation at 60°C. Poly A protected both activities slightly against heat inactivation. The true specificity of the enzyme is also shown by the finding that ADP but not dADP protected the enzyme against heat denaturation.

Disc gel electrophoresis in polyacrylamide: This also showed that the polymerizing activities utilizing ADP and dADP were not separable. Electrophoresis was carried out at pH 8.7 in 5% acrylamide gels. The gel was sliced, proteins were eluted from the slices with buffer, and the eluate was used for both dADP and ADP polymerization. Fig. 4 shows that both activities coincide.

Thus, at no stage of purification was there evidence of separation of these two activities. It was concluded that native polynucleotide phosphorylase of *E. coli* catalyzes the polymerization of deoxyribonucleoside diphosphates in the presence of Mn^{++} . Deoxyribonucleoside diphosphates are, however, poor substrate for the enzyme.

Effect of salt and temperature: During the early stage of enzyme purification, it was found that there was no detectable dADP polymerization activity after DEAE-cellulose chromatography if NaCl were not removed before the assay. The effect of NaCl on the dADP and ADP polymerization activities was investigated. Fig. 5 shows the marked effect of NaCl on both polymerization activities. dADP polymerization is inhibited about 90% by NaCl at 0.2M. In contrast, NaCl stimulated ADP polymerization about 4-fold at 0.4M NaCl. This observation suggested that a different form of the enzyme might be responsible for the dADP polymerization as compared with that for ADP polymerization.

The rates of the two reactions observed at various temperatures are shown in Fig. 6. It can be seen that dADP polymerization activity decreased sharply above 45°C, whereas that for ADP did not.

Lucas and Grunberg-Manago (17) have reported that the optimal temperature for the polymerization of ADP and GDP is about 60°C, while that for CDP and UDP is between 45 and 55°C. Thus, while the temperature effect may be interpreted in terms of an effect on the enzyme, it is equally plausible to explain

Fig. 4. Activities of dADP polymerization in samples from polyacrylamide gel electrophoresis.

100 μ g of enzyme was applied to each gel (5% acrylamide). Electrophoresis was at 4°C for 2 hours. The reaction was carried out at 37° for 60 min. The specific activities for ADP and dADP are respectively 7.7×10^2 and 3.5×10^3 cpm/nmole. ADP polymerization ($\square--\square--\square$); dADP polymerization ($o--o--o$).

Fig. 5. Effect of NaCl on the polymerization.

The assay conditions for dADP and ADP incorporation were as described in Methods, except for the addition of NaCl to the system. The concentration of ADP in this experiment was 10mM. The enzyme concentration was 50 μ g per assay. ADP polymerization ($\square$ -- $\square$ -- $\square$); dADP polymerization ($\blacktriangle$ -- $\blacktriangle$ -- $\blacktriangle$).

Fig. 6. Effect of temperature on dADP and ADP polymerization.

The reaction conditions were as described in the text. The incubation was for 20 minutes. The enzyme concentration was 50 μ g per assay. ADP polymerization ($\square$ -- $\square$ -- $\square$); dADP polymerization ($\blacktriangle$ -- $\blacktriangle$ -- $\blacktriangle$).

the different temperature stability in terms of the properties of the polynucleotide. The specific activity of the enzyme was determined at different protein concentrations when the activities were assayed at 37° and 50°C. Results are shown in Fig. 7. It was found that the specific activity of the enzyme does not vary with protein concentration in the range from 0.05 to 1.0 mg/ml, either in dADP or ADP polymerization, when assayed at 37°C. However, there was a parallel decrease in both specific activities at 50° when the enzyme concentration was higher than 0.6 mg/ml. The results appear to rule out the possibility that different polymeric forms of the enzyme are the active forms for dADP and ADP polymerization reaction.

Tryptic digestion of enzyme: Klee (18) was able to isolate primer-dependent polynucleotide phosphorylase from Micrococcus luteus after trypsin treatment. An attempt was made to study the effect of tryptic digestion on the catalytic activities of the E. coli enzyme towards dADP and ADP. As shown in Table II, the dADP polymerization activity is markedly decreased after the trypsin treatment while there was only 50% decrease in the ADP polymerization activity. Though the activities for these two substrates were differentially decreased, in neither case did trypsin produce a primer-dependent form of the enzyme (Table II).

Effect of NaCl on the additions of ppdA to pd(A₄) primer:

Attempts to limit the enzymatic reaction to the addition of a single nucleotide residue to the primer by reducing the concentration of enzyme and limiting the time allowed for reaction were not successful. In the incubation mixtures of optimal composition for rate of reaction either no product was formed or the product consisted of primer to which several nucleotide residues had been added. These results suggest that under these conditions primer is bound to the enzyme, accepts a nucleotide residue and the product, without dissociating from the enzyme, serves as primer for further addition. Under these conditions it would be necessary to mix equivalent portions of enzyme, primer and deoxynucleoside diphosphate to obtain acceptable yields of the desired product. As a more practical alternative

Fig. 7. Effect of enzyme concentration.

The assay conditions were essentially as described in Methods, except that the ADP concentration was 10 mM and the enzyme concentration was varied. The incubation time for ADP polymerization was 10 minutes and for dADP polymerization was 20 minutes.

TABLE II

Effect of trypsin treatment on the polymerization of
dADP and ADP.

Substrate	Polymerization activity (nmoles nucleotide incorporated/20 min)			
	Not treated		Trypsin treated	
dADP	2.14	2.8*	0.056	0.073*
ADP	31.9	27.0**	15.3	13.0**

* Assayed in the presence of 0.29 μ moles of $\text{pd}(\text{A}_3)$.

** Assayed in the presence of 0.9 μ moles of ApA .

Enzyme (4.7 mg) in 1 ml storage buffer was incubated with trypsin (12.5 μ g) at 25°C for 30 min, and the reaction was stopped by the addition of soyabean trypsin inhibitor (12.5 μ g). The enzyme was freed of trypsin, trypsin inhibitor and trypsin-inhibitor complexes by passing the reaction mixture through Sephadex G-100 column (1.2 x 60 cm) which had been pre-equilibrated with the storage buffer. The assay conditions were as described in Methods. The specific activity of dADP and ADP were 2.53×10^3 cpm/nmole and 1.26×10^3 cpm/nmole respectively. 10 mM MgCl_2 was used for ADP polymerization. The concentration of the enzyme used in the assay was 50 μ g.

we chose to examine the effects of ionic strength of the incubation mixture on the products. Sodium chloride, for example, in the case of polynucleotide phosphorylase, assists the dissociation of enzyme and product, thus tending to equalize the probability that each substrate molecule will interact with the enzyme (5,6). Table III shows the products obtained from addition of deoxyadenylyl residues from deoxyadenosine diphosphate to $pd(A_4)$ in the presence of several concentrations of sodium chloride. The extent of reaction decreased with increasing concentrations of salt. The proportion of higher addition products also decreased. In the presence of 0.6M sodium chloride the principal product was $pd(A_5)$. In this series of experiments the highest yield of the desired product was obtained in the solution containing 0.3M sodium chloride. This concentration was chosen for use in further studies. Obviously, where the addition of more than one nucleotide residue is required a different salt concentration would be chosen.

Phosphorolysis of primer. In using these methods for the controlled synthesis of oligodeoxynucleotides it is important to minimize the possibility of depolymerization of the primer. Our preparation of enzyme seem to be largely free of nuclease activity but phosphorolysis of the primer does occur. We have chosen to express the degree of phosphorolysis in a reaction adding one radioactive nucleotide residue to a primer containing n residues as the ratio of radioactivity in the product having n residues to that of the material of $n + 1$ residues, expressed as a percentage. Note that where every molecule of primer is degraded by one residue in this way, the figure would be 50%. As would be expected, the extent of phosphorolysis increased with the length of time of incubation, while the yield of the desired product decreased (Table IV). Phosphorolysis was therefore minimized by limiting the duration of the reaction.

DISCUSSION

Deoxynucleotidyl transferase from calf thymus has been used (19) to catalyze addition of deoxynucleoside triphosphates to a primer in order to prepare certain oligodeoxynucleotides. As a synthetic method this has the disadvantage that the pro-

TABLE III

Effect of NaCl on the addition of dADP to pd(A₄) primer.

NaCl (M)	Primer recovered (A _{260nm})	Products formed (A _{260nm})			
		pd(A ₅)	pd(A ₆)	pd(A ₇)	oligonucleotides*
0.05	6.86	3.15	1.89	0	8.64
0.1	6.4	3.85	1.85	0.56	5.49
0.2	5.2	4.10	3.15	1.95	4.16
0.3	4.5	4.3	2.85	1.67	0.94
0.6	7.5	3.0	0	0	0.8

* Oligonucleotides eluted by 1M (NH₄)₂CO₃. This fraction also contained polynucleotide.

The 1 ml solution which contained 0.32 μ mole pd(A₄); 0.05 M Tris-HCl (pH 8.5); dADP 1.5×10^{-3} M; MnCl₂ 0.01 M; 2-mercapto-ethanol 0.015 M; and enzyme 1.5 mg was incubated at 37°C for 10 hrs with various concentrations of NaCl. The reaction mixtures were analyzed on a DEAE-cellulose column (1.2 x 10 cm) in the carbonate form. The column was eluted at 25° using a linear gradient of ammonium bicarbonate (400 ml of water and 400 ml of 0.4 M NH₄HCO₃) at a flow rate of approximately 0.7 ml/minute.

TABLE IV

Phosphorolysis of primer at different incubation times

Time of incubation (hrs.)	Primer recovered pd(A ₄) μ mole *	Products formed		Phosphorolysis of recovered pd(A ₄) **
		pd(A ₅) μ mole	pd(A ₆) μ mole	
3		0.110	0.033	1.2%
6		0.063	0.039	3.0%
12		0.057	0.04	6.4%

* The initial amount of pd(A₄) was 0.32 μ moles. The reaction was carried out at 37° in 0.3M NaCl with other components as described under Methods.

** The extent of phosphorolysis was estimated by expressing the amount of radioactive pdA incorporated into the pd(A₄) as a percentage of the amount of recovered pd(A₄).

duct is not a single product but a series of oligonucleotides of varying chain length. The yield of the desired product is thus rather low. The experiments reported here used a different enzyme to perform the same type of reaction, i.e. addition of a deoxynucleotide residue to an oligonucleotide primer. Under the conditions selected we have been able to obtain as the predominant product the oligodeoxynucleotide containing a single added nucleotide residue. Thus stepwise synthesis is made possible. This enzymatic synthesis occurs rapidly in aqueous solution, facilitating recovery of the product. No protecting groups are required on the primer or the residue to be added, simplifying the procedure as compared with chemical methods of synthesis.

The enzyme preparation used here was from E. coli B. Initial preparation followed the procedure described by Hsieh (2) for a similar activity from E. coli W3110 polA₁. The possibility that polymerization of deoxynucleoside diphosphates was due to the breadth of the substrate specificity of E. coli polynucleotide phosphorylase (20) was confirmed by the finding that throughout the purification of the enzyme the two activities are inseparable. Since the rate of polymerization is greatly dependent on the concentrations of both nucleoside diphosphates and metal ion, the relative rate of ADP and dADP polymerization varied with respect to the experimental conditions used. However, at all stages the polymerization of ribonucleoside diphosphates was much greater than that of deoxynucleoside diphosphates. In the presence of high salt concentration or after mild treatment with trypsin the activities were differentiated from each other. Both these treatments might be expected to alter conformations around the active site of the enzyme, perhaps favouring polymerization of one class of substrate over the other.

In general, polynucleotide phosphorylase from Micrococcus luteus possesses similar properties to polynucleotide phosphorylase from E. coli, though tryptic degradation of the M. luteus enzyme markedly affects its properties with regard to requirements for a primer. We have used the M. luteus enzyme for the synthesis of deoxyoligonucleotides (21). It was found that under the same reaction conditions, this enzyme also adds a single

deoxyribonucleotide residue to a deoxyoligonucleotide primer but with low yield (0.2-1%). A similar yield was obtained with the primer-dependent enzyme. The reason for the low yield is probably the lower specific activity of the pure M. luteus polynucleotide phosphorylase as compared with that from E. coli (5). In addition, the M. luteus enzyme seems to have higher substrate specificity for ribonucleoside diphosphates (21). Possibly the yield can be improved by using very high enzyme concentration or different reaction conditions.

Because the enzyme is similar in properties to polynucleotide phosphorylase, it was logical to try the method used by Thach (6) for synthesis of ribo-oligonucleotides. The basis of this method is the use of high concentrations of salt to inhibit polymerization. The results reported here indicate that this approach is successful and also that the problem of phosphorylation of primer is minimal (6). The following paper (7) describes the application of this method to the synthesis of a variety of oligodeoxyribonucleotides.

* Research supported by the Medical Research Council of Canada

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