'Single addition' and 'transnucleotidation' reactions catalyzed by polynucleotide phosphorylase. Effect of enzymatic removal of inorganic phosphate during reaction

J.J.Sninsky, G.N.Bennett, and P.T.Gilham

Biochemistry Division, Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907, USA

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ABSTRACT

The reaction of the tetranucleotide, pA-A₂-A, with 2'(3')-O-(α -methoxyethyl)uridine 5'-diphosphate, Mg²⁺ ions, and *M. luteus* polynucleotide phosphorylase followed by mild acid treatment to remove the blocking groups results in a 49% yield of the desired single addition product, pA-A₃-U, together with smaller amounts of pA-A-U, pA-A-A, pA-A₂-U, pA-A₂-A, pA-A₃-A, pA-A₄-U, and pA-A₄-A. The side products are thought to arise from the phosphorylase of the acceptor molecule by the inorganic phosphate formed in the reaction mixture and from subsequent additions to the various oligonucleotide species by the resulting adenosine 5'-diphosphate. A system developed for the removal of inorganic phosphate as it is formed in the synthesis involves the addition to the reaction mixture of calf spleen nucleoside phosphorylase and nicotinamide riboside and, under these conditions, pA-A₃-U can be prepared in 90% yield with essentially no side products. Under similar conditions, pA-A₃-A, pA-A₃-G, and pA-A₃-C may be prepared from pA-A₂-A and the appropriate blocked nucleoside diphosphate in yields of 85-94%. The incubation of pA-A₂-A alone with polynucleotide phosphorylase exhibits the phenomenon of "transnucleotidation" in that the molecule is partially converted to oligonucleotides of smaller and larger chain lengths. In the presence of the phosphate removal system, however, the tetranucleotide is not attacked by the enzyme, and thus, "transnucleotidation" appears to be simply a combination of phosphorolytic and addition reactions catalyzed by trace amounts of inorganic phosphate contaminating the enzyme and/or the substrate.

INTRODUCTION

The method for the synthesis of oligoribonucleotides of defined sequence that has recently been developed in this laboratory involves the use of polynucleotide phosphorylase to catalyze the successive addition of single nucleotide moieties to an acceptor oligonucleotide.^{1,2} The synthetic system employs monomers (2'-O-(α -methoxyethyl)nucleoside 5'-diphosphates) containing a chemical blocking group that permits the enzymatic addition of single nucleotide units to the acceptor oligonucleotide and prevents the subsequent addition of further nucleotide residues. The blocking group can be removed from the terminus of the oligonucleotide product to yield a new acceptor molecule that is then available for further single addition reactions. The success of this procedure in terms of its ability to produce high yields of

specific oligonucleotides depends to some extent on the conservation of the structure of the acceptor molecule during the addition reactions. Recent experiments have shown that, in some cases, products other than the desired oligonucleotide can be formed and this effect results in a reduced yield of the desired sequence as well as increased difficulty in carrying out its purification. The side products apparently arise from the rearrangement of a portion of the acceptor molecules during the synthetic step. This rearrangement appears to be related to the phenomenon of "transnucleotidation", a concept that was introduced by Singer *et al.*³ to account for the enzyme catalyzed rearrangement of oligonucleotides into smaller and larger species without the intervention of inorganic phosphate or nucleoside 5'-diphosphates. The present work shows that both the rearrangement of oligonucleotide acceptors during stepwise synthesis and the "transnucleotidation" of oligonucleotides are prevented by the use of an ancillary enzyme system to effect the removal of inorganic phosphate either present or formed in the reaction mixture.

EXPERIMENTAL SECTION

Materials. β -Nicotinamide adenine dinucleotide was purchased from Sigma Chemical Co., St. Louis, Mo. *M. luteus* polynucleotide phosphorylase and calf spleen nucleoside phosphorylase were obtained from Boehringer Mannheim Corp., New York, N.Y. and a unit of activity of the polynucleotide phosphorylase is defined as the amount required to polymerize 1 μ mol of UDP in 15 min at 37° under the assay conditions specified by Matthaai *et al.*⁴ *E. coli* alkaline phosphatase (Grade, BAPF) and snake venom phosphodiesterase (Grade, VPH) were purchased from Worthington Biochemical Corp., Freehold, N.Y. The phosphatase was dialyzed against 5 mM Tris-Cl, pH 8 prior to use, and a unit of activity is defined as the quantity required to liberate 1 μ mol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min at 25° (Garen and Levinthal⁵). The chromatographic materials, Dowex 1-X2, Dowex 2-X8, and beaded polyacrylamide, were purchased as AG 1-X2 (-400 mesh), AG 2-X8 (100-200 mesh) and Bio-Gel P-2 (200-400 mesh), respectively, from Bio Rad Laboratories, Richmond, Calif. The mono- α -methoxyethyl derivatives of the four ribonucleoside 5'-diphosphates were prepared as previously described.^{1,2} The adenosine oligonucleotides were obtained from the fractionation of the products resulting from the degradation of poly(A) with pig liver nuclease according to the method of Bennett *et al.*² The products pA-A-A, pA-A₂-A, pA-A₃-A, and pA-A₄-A which were purified by ion-exchange chromatography as described below had retention

volumes of 42, 57, 78, and 99 ml respectively. In addition, pA-A₂-A was characterized by alkaline hydrolysis as described below to yield the products, pAp, Ap, and A, in the molar ratio of 1.0:1.99:0.91, respectively.

Nucleoside Phosphorylase. The commercial nucleoside phosphorylase preparation (5 mg, suspended in 1 ml 3.2 M ammonium sulfate) was diluted to 2 ml with 0.01 M glycine pH 9 (20°) and passed through a column (1 X 40 cm) of Bio-Gel P-2 beaded polyacrylamide using the same buffer. The sulfate-free enzyme was collected in a volume of about 8 ml and was shown to contain about 14 units of activity. The following assay of the enzyme was developed with conditions that approximate those used in the synthetic reaction. The assay solution (total volume 1.0 ml) contained 1.0 M glycine, pH 10 at 20° (100 µl), 0.1 M MgCl₂ (100 µl), 0.01 M potassium phosphate (10 µl), nicotinamide riboside (0.2 µmol) and nucleoside phosphorylase (5 µl). The mixture was kept at 25° and the decrease in absorbance at 265 nm was measured against time with the same mixture, less the riboside and enzyme, in the reference cell. The conversion of nicotinamide riboside to nicotinamide was calculated using $\epsilon_{265\text{nm}}$ values of 4600 and 2700 respectively. Since the riboside hydrolyzes slowly in alkaline solutions, a control experiment, in which no enzyme was added, was also carried out to make a correction for this reaction. The unit of activity is defined as the amount of enzyme that converts 1 µmol of the riboside to nicotinamide per min. at 25°.

Nicotinamide Riboside. Nicotinamide riboside was prepared by a modification of the method of Kaplan and Stolzenbach.⁶ Nicotinamide adenine dinucleotide (100 mg) was dissolved in 2 ml of a solution of 0.12 M Tris-Cl-0.03 M MgCl₂, pH 8.7 containing snake venom phosphodiesterase (5 mg) and alkaline phosphatase (9 units), and the mixture was incubated at 37° for 65 h. The mixture was then brought to pH 1.5 with concentrated HCl and heated at 100° for 3 min. The precipitate was removed by centrifugation and the supernatant solution was applied to 150 cm of Whatman No. 3MM chromatographic paper. Descending elution with *n*-butanol-30% acetic acid (2:1, v/v) gave bands with R_F values of 0.01, 0.18, 0.39, and 0.70 corresponding to the products, NAD, nicotinamide riboside, adenosine, and nicotinamide, respectively. The nicotinamide riboside, which was obtained in 51% yield, was extracted with water and passed through a column (1 X 10 cm) of Dowex 2-X8 (Cl⁻ form) ion-exchange resin to remove any contaminating inorganic phosphate, and finally concentrated in vacuo. A portion (1.0 ODU_{254nm}) was shown to be free of phosphatase activity by incubation at 37° for 12 h. in a solution of 0.025 mM *p*-nitrophenyl phosphate in 1.0 M Tris-Cl, pH 8 (1 ml).

Addition of Nucleotides to Adenosine Tetranucleotide. A solution of nucleoside phosphorylase (0.18 units) in 100 μ l of 0.01 M glycine, pH 9 at 20°, was lyophilized and added to a mixture of 1.0 M glycine, pH 10 at 20° (10 μ l), 0.1 M $MgCl_2$ (10 μ l), 2'-(3')-O-(α -methoxyethyl)uridine 5'-diphosphate (10 μ l, 1.0 μ mol), nicotinamide riboside (1.2 μ mol in 10 μ l of water), and 40 μ l of polynucleotide phosphorylase (30 units/ml). The mixture was incubated at 37° for 30 min. and then treated with adenosine tetranucleotide (3.9 ODU_{254nm}, 78 nmol in 10 μ l of water) and nucleoside phosphorylase (10 μ l, 0.018 unit). The reaction was allowed to proceed at 37° for 4.5 h. with additions of nicotinamide riboside (5 μ l, 0.6 μ mol) at one hour and nucleoside phosphorylase (10 μ l, 0.018 unit) at 1, 2, and 3 h. after the addition of the tetranucleotide. The mixture was brought to about pH 2.1 by addition of 1.0 M HCl (15 μ l) and kept at 37° for 1 h. to remove the methoxyethyl groups. The reaction mixture was then treated with 1.0 M NH_4OH (15 μ l) and subjected to chromatographic separation by the ion-exchange method described by McCutchan and Gilham.⁷ Separation was effected on a column (100 X 0.4 cm) of Dowex 1-X2 by elution under pressure at 12 ml/h. with 200 ml of 40% ethanol containing a linear gradient of 0.3 - 0.5 M NH_4Cl , pH 8. The elution pattern is shown in Figure 1B. Similar syntheses were carried out with the 2'-O-(α -methoxyethyl) derivatives of the diphosphates of adenosine (0.28 μ mol), cytidine (0.31 μ mol), or guanosine (0.21 μ mol) in place of the modified uridine diphosphate. The chromatographic retention volumes of these products were pA-A₃-C, 73 ml; pA-A₃-U, 77 ml; pA-A₃-A, 77 ml; pA-A₃-G, 95 ml. In order to demonstrate the effect of phosphate removal, a reaction, similar to that described above for methoxyethyluridine diphosphate, was carried out except that, in place of the nicotinamide riboside and nucleoside phosphorylase, appropriate amounts of water were added. Analysis as described above gave the elution pattern shown in Figure 1A.

The yields of the various synthetic products were determined by using calculated ϵ_{254nm} values. In the case of pA-A₃-U the ϵ_{254nm} in 0.3 M NH_4Cl - 40% ethanol was determined by comparing the absorbance of a specific quantity of the oligonucleotide in that solvent with the calculated molar amounts of its components (pAp, Ap, U) determined by subjecting an equal quantity of the oligonucleotide to alkaline hydrolysis. The corresponding values for pA-A₃-N where N = A, C, or G were determined by subtracting the ϵ_{254nm} of the uridine moiety from the ϵ_{254nm} of pA-A₃-U and adding the ϵ_{254nm} value for adenosine, cytidine, or guanosine respectively. The derived ϵ_{254nm} (0.3 M NH_4Cl - 40% ethanol) values were pA-A₃-U, 59,000; pA-A₃-A, 64,500; pA-A₃-C, 56,900;

pA-A₃-G, 63,900.

Reaction of Adenosine Tetranucleotide with Polynucleotide Phosphorylase.

A mixture of 40 μ l of polynucleotide phosphorylase (30 units/ml), 1.0 M glycine, pH 10 at 20° (10 μ l), 0.1 M MgCl₂ (10 μ l), nicotinamide riboside (1.2 μ mol in 10 μ l of water), and nucleoside phosphorylase (50 μ l containing 0.09 units) was incubated at 37° for 45 min and then mixed with pA-A₂-A (2 ODU_{254nm} in 5 μ l of water). The incubation was allowed to proceed for a further 2 h. and the reaction mixture was then subjected to ion-exchange chromatography as described above. The control experiment was similar to the above except that the appropriate amount of water was added in place of the nicotinamide riboside and nucleoside phosphorylase.

Characterization of Oligonucleotide Products. The fractions corresponding to each product indicated in the elution patterns of Figure 1 were combined and concentrated in vacuo to a small volume, and were desalted by passing them through a column (1 X 60 cm) of Bio-Gel P-2 beaded polyacrylamide using 20% ethanol as the eluting solvent. A portion of each product (2-4 ODU_{254nm}) was evaporated to dryness and treated with 0.25 M NaOH (0.3 ml) for 20 h. at 37°. The products of the alkaline hydrolysis were analyzed by the ion-exchange procedure of Ho and Gilham.⁸ The column of Dowex 1-X4 had dimensions, 110 X 0.2 cm and the following additions were made, in order, via a sample injection loop: 0.3 ml of 1 M NaOH, 2.0 ml of 20% ethanol, the alkaline hydrolysate, and 1.0 ml of 20% ethanol. Separation was effected by elution, under pressure, at 8 ml/h. with 200 ml of 20% ethanol containing a linear gradient of 0 - 0.5 M NH₄Cl which had been adjusted to pH 10 with ammonia. In this system, adenosine, 2'-O-(α -methoxyethyl)uridine, uridine, adenosine 2'-phosphate, adenosine 3'-phosphate, and adenosine 2'(3'), 5'-diphosphate have retention volumes of 31.5, 33.6, 35.7, 69.3, 73.5, and 92.4 ml respectively. The results of the analyses are listed in Table 1.

RESULTS

For the study of the conservation of the acceptor molecule during stepwise synthesis the analysis of the single addition of uridine phosphate residues to adenosine tetranucleotide was chosen because, in this case, the particular analytical system used fractionates the various oligonucleotide products according to their chain lengths and permits the quantitative analysis of all the species present at the end of the reaction period. The tetranucleotide, pA-A₂-A, and 2'(3')-O-(α -methoxyethyl)uridine 5'-diphosphate were exposed to polynucleotide phosphorylase in the presence of Mg²⁺ ions. The blocking groups were then removed and the products were separated by

Figure 1: Elution patterns from the chromatographic analysis of the products from the reaction of pA-A₂-A with 2'(3')-O-(α -methoxyethyl)uridine 5'-diphosphate and polynucleotide phosphorylase (A), and the products from a similar reaction containing, in addition, nucleoside phosphorylase and nicotinamide riboside (B). Separation was effected on a column (100 X 0.4 cm) of Dowex 1-X2 ion-exchange resin by elution at 12 ml/h., under pressure, with 200 ml of 40% ethanol containing a linear gradient of 0.3-0.5 M ammonium chloride, pH 8.0. Peak identification: 1, nicotinamide and nicotinamide riboside; 2, uridine 5'-diphosphate; 3-7, oligonucleotide products.

Table I: Characterization of Products Corresponding to Peaks in Elution Patterns of Figure 1.

Elution Pattern	Peak	$\bar{V}^a$	Oligonucleotide Species	pAp:Ap:U:A ^b	% U Terminal
A	3	42	pA-A-U + pA-A-A	1.0:1.22:0.13:0.87	13
	4	57	pA-A ₂ -U + pA-A ₂ -A	1.0:2.21:0.66:0.35	65
	5	78	pA-A ₃ -U + pA-A ₃ -A	1.0:2.97:0.81:0.10	89
	6	99	pA-A ₄ -U + pA-A ₄ -A	1.0:4.27:0.78:0.07	92
B	5	78	pA-A ₃ -U	1.0:2.97:0.95:<0.02	>98

^aChromatographic retention volumes on a column (100 X 0.4 cm) of Dowex 1-X2 ion-exchange resin by elution at 12 ml/h. with 200 ml of 40% ethanol containing a linear gradient of 0.3 - 0.5 M NH₄Cl (pH 8.0). ^bMolar ratios of products obtained by alkaline hydrolysis.

ion-exchange chromatography (Figure 1A). The various oligonucleotides corresponding to the peaks in the elution pattern were desalted and then subjected to alkaline hydrolysis. This method of analysis allows the determination of both the chain lengths and the identity of the 5'- and 3'- terminal nucleotides of the molecules in each isoplith fraction (Table I). The desired product, pA-A₃-U, was obtained in 49% yield based on the amount of tetranucleotide added to the reaction mixture, and this yield corresponds to 89% of the total pentanucleotides formed. The structures of the other products formed are shown in Table I.

Preliminary studies on nucleoside phosphorylase as a system for the *in situ* removal of phosphate showed that, in the presence of nicotinamide riboside, the enzyme was capable of quantitatively removing the phosphate anion from solution with the formation of ribose 1-phosphate and nicotinamide. Figure 1B shows the elution pattern from the analysis of a reaction similar to that described above for pA-A₂-A and the blocked uridine diphosphate but containing, in addition, nucleoside phosphorylase and nicotinamide riboside. The main product, pA-A₃-U was obtained in 90% yield and with a purity of over 98% (Table I). Similar reactions between adenosine tetranucleotide and the 5'-diphosphates of the 2'-O-(α -methoxyethyl) derivatives of adenosine, cytidine, and guanosine were also performed in the presence of the phosphate removal system. In each case, the elution pattern from the chromatography of the products was similar to that shown in Figure 1B, and the yields obtained

for the products, pA-A₃-A, pA-A₃-C, and pA-A₃-G were 85%, 86%, and 94%, respectively.

The action of polynucleotide phosphorylase and Mg²⁺ towards pA-A₂-A alone was also studied under similar conditions. After incubation for two hours (about half the reaction time used in the syntheses described above) the products were separated by column chromatography and shown to consist of pA-A-A (18%), pA-A₂-A (53%), and pA-A₃-A (13%), together with smaller amounts of longer oligonucleotides. In a second experiment the polynucleotide phosphorylase was first preincubated with nucleoside phosphorylase and nicotinamide riboside prior to the addition of the tetranucleotide. Analysis of this mixture after a two hour reaction period showed that the pA-A₂-A was totally conserved.

DISCUSSION

The variety of oligonucleotide sequences that were formed from the reaction of pA-A₂-A with methoxyethyluridine diphosphate (UmeDP) are considered to have arisen from the action of the inorganic phosphate released during the reaction. These products are presumed to derive from the phosphorylase of the tetranucleotide followed by the reaction of the liberated adenosine diphosphate with the various oligonucleotide species according to the following equations:

It was considered that the removal of the phosphate anion as it is formed in these syntheses would prevent the production of undesired sequences by eliminating reactions corresponding to equations 2-5. The choice of nucleoside phosphorylase and the substrate, nicotinamide riboside, as the phosphate removal system was made on the basis of some studies carried out by Rowen and Kornberg⁹ on a nucleoside phosphorylase activity from pig liver. The enzyme converts nicotinamide riboside to nicotinamide, ribose 1-phosphate, and H⁺ ions and thus, in solutions of neutral or slightly alkaline pH, the system is capable of completely removing the phosphate anion. The inclusion of the phosphate removal system in the synthesis of pA-A₃-N (where N = A, C, G, or U) results in a high yield of the desired product with essentially no contamination by products containing rearranged sequences.

In addition, the continuous removal of phosphate allows syntheses to be performed in which there is a relatively low ratio of blocked nucleoside diphosphate to acceptor molecule, a factor that should be of some economic importance in the synthesis of larger and more complex oligonucleotides.

The present work also shows that, in the presence of Mg^{2+} ions, only single addition is permitted with blocked nucleoside diphosphates. Earlier studies in this laboratory employed Mn^{2+} as the divalent ion because, in the absence of an ancillary system for the removal of phosphate, this ion seemed to effect less phosphorolysis and rearrangement of the acceptor molecule. However, recent experiments have shown that, in contrast to Mg^{2+} , Mn^{2+} allows a small amount of double addition of blocked nucleotides to the acceptor. A study of the comparison of the effects of the two divalent cations in these syntheses will be the subject of a forthcoming publication. It should also be pointed out that, even in the absence of the phosphate removal system, there is no difficulty in preparing high yields of tetranucleotides by single addition to trinucleotides. These syntheses, however, should be considered as special cases since the relative lack of rearrangement of the acceptor molecule undoubtedly arises from the fact that trinucleotides are relatively poor substrates for the phosphorolytic reaction.¹⁰

The concept of "transnucleotidation" was originally introduced³ to account for the observation that polynucleotide phosphorylase appeared to catalyze the transfer of nucleoside phosphate moieties from one oligonucleotide to another without the intervention of inorganic phosphate or nucleoside diphosphate. Thus, on incubation with the enzyme, the trinucleotide, pA-A-A, was observed to rearrange to a mixture of pA-A_n-A (where n = 0, 1, 2, 3). In the present work, an equivalent rearrangement of pA-A₂-A was observed, and was subsequently shown to be completely inhibited in the presence of nucleoside phosphorylase and nicotinamide riboside. It is concluded that this chain length rearrangement arises from the presence in the enzyme or substrate of a small amount of inorganic phosphate that catalyzes phosphorolysis and addition reactions via the formation of the nucleoside 5'-diphosphate.

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