# IDENTIFICATION OF THE NUCLEOSIDE MONOPHOSPHATE END-GROUP ON THE PRODUCT OF THE POLYNUCLEOTIDE PHOSPHORYLASE REACTION

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Polynucleotide phosphorylase has been shown to catalyze the polymerization of a number of nucleoside diphosphates yielding linear polymers composed of ribonucleotide units linked by 3'-5' phosphodiester bridges (Grunberg-Manago, 1962). In the case of the synthesis of polyadenylic acid by the enzyme from Escherichia coli, chain initiation occurs de novo without the participation of polynucleotide primers that may possibly contaminate the enzyme preparation (Williams and Grunberg-Manago, 1964). The simplest mechanism for the chain initiation reaction involves bond formation between the 3' hydroxyl group of one diphosphate molecule and the $\alpha$-phosphate of a second molecule of nucleoside diphosphate, in a reaction analogous to that proposed for RNA polymerase (Maitra, Novogrodsky, Baltimore and Hurwitz, 1965). This mechanism predicts that the 5'-end group of the polymer synthesized by polynucleotide phosphorylase would terminate with a 5'-pyrophosphate group; that is, the polymerization of NDP would be expected to yield a polymer with the structure ppNpNp...NpNpN (N being an unspecified nucleoside; NDP an unspecified nucleoside diphosphate).

The present paper, however, provides evidence that ribonucleotide polymers synthesized by polynucleotide phosphorylase terminate with a 5-monophosphate rather than a 5'-pyrophosphate. The possible significance of these findings on the mechanism of the enzyme is discussed.

Polynucleotide phosphorylase was purified from E. coli by the methods of Williams and Grunberg-Manago (1964). Uridine-3'-phosphate-5'-pyrophosphate (ppUp) and uridine-3',5'-diphosphate

(pUp) were synthesized according to Michelson (1964). ($\beta$-$^{32}$P) ADP and ($\beta$-$^{32}$P) UDP were prepared from $^{32}P_i$ and unlabeled nucleoside diphosphate, using the exchange reaction catalyzed by polynucleotide phosphorylase (Grunberg-Manago, Ortiz and Ochoa, 1956). Five times recrystalized ribonuclease and tritiated uridine diphosphate were products of Sigma Chemical Company and Schwarz BioResearch, respectively. Radioactivity measurements were made in either a Packard Tri-carb scintillation counter or a Tracerlab low background counter.

The nature of the 5'-end group on the product of the polynucleotide phosphorylase reaction was first investigated by alkaline degradation of polyuridylic acid synthesized from UDP. A reaction medium containing doubly labeled UDP (see Table I) was incubated at 37°. Aliquots (0.15 ml) were removed after 6 and 60 minutes of incubation and were added to 1.5 ml of a solution containing 0.01 M ammonium acetate-0.001 M sodium dodecyl sulfate. The product of the reaction, polyuridylic acid, was separated from the low molecular weight components of the reaction mixture by gel filtration on Sephadex G-50 equilibrated with 0.01 M ammonium acetate. After lyophilyzation the polymer was hydrolyzed in 0.3 M KOH for 18 hours at 37°. The alkaline solution was neutralized with Dowex 50 ($H^+$) and unlabeled pUp and ppUp were added as chromatographic markers. The components of the hydrolysate were separated on Dowex 2, using stepwise elutions with increasing concentrations of HCl and KCl. Since alkaline cleavage of the phosphodiester bond yields exclusively 2'(3') mononucleotides, ppUp, Up and U would be the expected hydrolysis products if the polyuridylic acid terminated with a 5'-pyrophosphate group; in contrast, a polymer with a 5'-monophosphate end group would yield pUp, Up and U on alkaline digestion. Table I shows the observed distribution of tritium and radioactive phosphorous in Up, pUp and ppUp. Negligible radioactivity was found in uridine-3'-phosphate-5'-pyrophosphate after either 6 or 60 minutes of incubation; however, the uridine-3',5'-diphosphate fractions were strongly labeled with tritium.

It is unlikely that the absence of ppUp is due to chemical degradation during the alkaline hydrolysis since authentic ppUp is not appreciably cleaved under the conditions employed. Alternatively, the terminal monophosphate group might be explained

TABLE I

COMPOSITION OF ALKALINE HYDROLYSATE OF POLYURIDYLIC ACID

| Dowex fraction | Isotope counted | Counts/min/0.15 ml incubation medium 6 min incubation | 60 min incubation |
|---|---|---|---|
| Up | $^{3}H$ | 615,000 | 1,060,000 |
| | $^{32}P$ | 25 | 30 |
| pUp | $^{3}H$ | 3,200 | 4,500 |
| | $^{32}P$ | 10 | 20 |
| ppUp | $^{3}H$ | 12 | 15 |
| | $^{32}P$ | 15 | 20 |

The reaction mixture (0.5 ml) contained 25 μmoles of Tris HCl (pH 8.2), 10 μmoles of doubly labelled UDP ($\beta$-$^{32}P$,$^{3}H$) (specific activity 8.1 x $10^6$ and 1.8 x $10^6$ counts/min/μmoles for $^{32}P$ and $^{3}H$, respectively), 5 μmoles $MgCl_2$ and 19 μg of polynucleotide phosphorylase (specific activity 340).

by the presence of an extraneous phosphatase activity in the polynucleotide phosphorylase preparation. Conceivably such a contaminating enzyme could hydrolyze the terminal pyrophosphate of the nascent polymer, yielding polyuridylic acid with a 5'-monophosphate group. This possibility seems unlikely since the polynucleotide phosphorylase preparation exhibited no phosphatase activity toward a variety of compounds, including ppUp, pUp or pppU under conditions identical to those employed in the polymerization reaction. To eliminate the possible effect of a phosphatase specific for a pyrophosphate linkage at the 5' end of a ribonucleotide polymer, an experiment was performed in which ribonuclease (10 μg/ml) was added to an incubation medium (Tris-HCl 50 μmoles pH 8.2; UDP, 20 μmoles; $MgCl_2$, 2 μmoles; enzyme 30 μg, specific activity 460; final volume 2 ml.). Under these conditions, the newly synthesized polyuridylic acid is immediately hydrolyzed upon release from polynucleotide phosphorylase; no oligonucleotide products due to incomplete degradation by ribonuclease were detectable during the course of the reaction. Thus, the 5' terminal group of the polymer was present as a mononucleotide during the

synthetic reaction and, therefore, would not be susceptible to attack by a phosphatase requiring a polymeric substrate. After incubation at 37° for 2 hours, the entire reaction mixture was streaked on Whatman HR paper and developed overnight in n-propanol:ammonia:water (50:10:35,v/v/v). Examination of the chromatogram under an ultraviolet lamp revealed a compound which migrated in parallel with authentic pUp, but no detectable material was found in the region where authentic ppUp migrates. The compound, tentatively identified as pUp, was eluted and subjected to paper electrophoresis (0.05 M sodium citrate, pH 3.5) where it traveled at the same rate as authentic pUp. Similar experiments in which ($^{14}$C) UDP was polymerized in the presence of ribonuclease and unlabeled ppUp resulted in no incorporation of radioactivity into the added uridine-3'-phosphate-5'-pyrophosphate which could be quantitatively recovered from the incubation. These results again indicate the presence of a monophosphate group at the initiation point of the polyuridylic acid chains.

Similar results have been obtained employing a purine derivative as substrate. Polymerization of ($\beta$-$^{32}$P) ADP yielded a high molecular weight polyadenylic acid which was virtually unlabeled. Further, $^{14}$C-adenosine-3',5'-diphosphate has been tentatively identified after alkaline hydrolysis of $^{14}$C-polyadenylic acid (Williams and Grunberg-Manago,1964). Thus, the absence of a 5'-pyrophosphate group in the polymerization product of the polynucleotide phosphorylase reaction appears to be a fundamental aspect of the enzymic mechanism.

The chain initiation reaction does not involve a free nucleoside monophosphate since the addition of (2-$^{14}$C) uridine-5'-monophosphate to a reaction medium containing unlabeled uridine diphosphate does not lead to incorporation of radioactivity into the terminal positions of polyuridylic acid (Godefroy,1966). It is possible that the chain initiation and elongation reactions both involve an enzymically catalyzed attack of a hydroxyl group on the $\alpha$-phosphate of the nucleoside diphosphate. For chain elongation the 3' hydroxyl of the growing polymer would be the nucleophilic group; in the case of chain initiation, however, the hydroxyl may come from the aqueous reaction medium resulting in an enzyme-nucleoside monophosphate complex. Subsequent reaction of the 3'-OH of the bound nucleoside monophosphate with a second molecule of nucleoside diphosphate would yield pNpN which could

then act as a nucleus for further chain elongation.

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