THE EFFECT OF TEMPERATURE ON POLYNUCLEOTIDE PHOSPHORYLASE FROM E.COLI*

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Polynucleotide phosphorylase isolated from Escherichia coli has been purified some 300-fold (Williams and Grunberg-Manago, 1964). This preparation appears to be free of nuclease, phosphatase and kinase activities and does not require a primer for polymerization of the four common nucleoside diphosphate substrates under optimal conditions (Williams and Grunberg-Manago, 1964). The enzyme obtained by the procedure of Williams and Grunberg-Manago has been purified 2-fold further by sucrose density gradient centrifugation (Thang and Thang, 1964). In this communication, we wish to report on the stability of this 600-fold purified enzyme to heat in the presence and absence of substrate, and also on the effect of temperature on the rates of polymerization and phosphorolysis.

Fig. 1 shows that polynucleotide phosphorylase from E. coli is heat stable below 55° under the conditions used in these experiments. At higher temperatures, there is a precipitous fall in enzymatic activity and nearly 100% inhibition is found at 65°.

The inactivation of the enzyme with time appears to proceed in two steps (Fig. 2); the initial rate of inactivation is rapid, but decreases after four minutes, as shown in the bottom curve. The inactivation appears to be non-reversible, and the degree of inhibition depends on the concentration of the enzyme used during the preincubation period, a more dilute solution of enzyme being more heat-labile.

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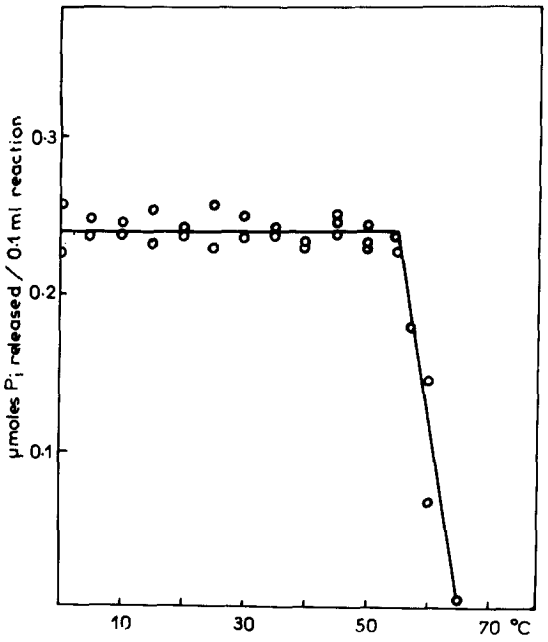

Fig. 1 The Stability of Polynucleotide Phosphorylase to Heat. 1.5 μg of enzyme (S.A., 330 μmoles P_i released/hr/mg enzyme) was preincubated at different temperatures for 10 minutes in 0.08 ml of a mixture containing 0.12 μmoles EDTA and 12 μmoles Tris buffer, pH 8.2. The mixture was then cooled to 0° and 1.7 μmoles UDP and 0.9 μmoles $MgCl_2$ were added, bringing the final volume to 0.1 ml. The resulting mixtures were incubated at 37° for 30 minutes and the reaction stopped with 2.5% perchloric acid. The reaction was followed by the release of inorganic phosphate (Fiske and Subbarow, 1925).

The effect of temperature on the rates of polymerization and phosphorolysis was also investigated. As demonstrated in Fig. 3A, the optimal temperature for the polymerization of ADP and GDP is about 60°, while that for CDP and UDP is between 45 and 55° The rate of reaction approximately doubles for every 10° change in temperature Fig. 3B shows that the optimal temperatures for the phosphorolysis of poly A, poly C and poly U are also high : a sharp optimum for poly A and poly C occurs at about 60°, and a rather broad peak was observed between 50 and 55° for poly U.

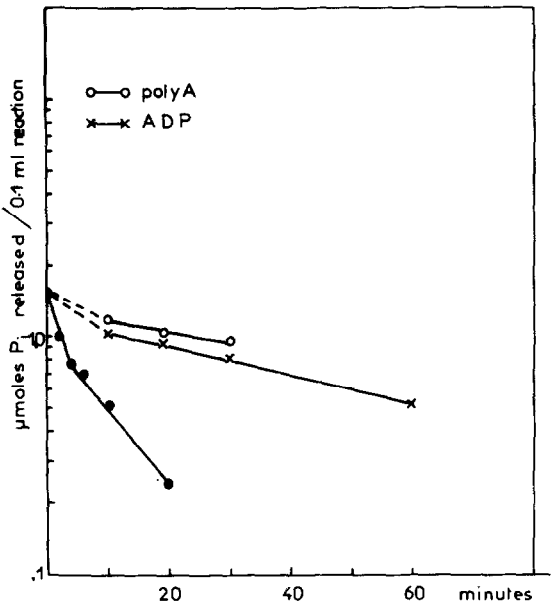

Fig.2 The Rate of Inactivation of Polynucleotide Phosphorylase at 60°

The experimental conditions were the same as for Fig.1, except that 0.18 μmoles poly A (o—o) or 1.8 μmoles ADP (x—x) were added in the appropriate cases. Before determination of residual activities, 0.9 μmoles $MgCl_2$, 0.18 μmoles poly A and 1.8 μmoles ADP were added as required, so that the final composition of all the reaction mixtures was identical.

Polynucleotide phosphorylase, preincubated alone, begins to lose activity at temperatures above 55°, whereas the optimal temperature for the polymerization of ADP and GDP and the phosphorolysis of poly A and C is about 60°. These results suggest that the enzyme is being protected against heat inactivation by the nucleoside diphosphate, the polymer, or both. Figs.2 and 4 show that both the nucleoside diphosphate and the polymer are able to protect the enzyme. As can be seen in Fig.2, the rate of inactivation is considerably decreased in the presence of either poly A or ADP. Furthermore, Fig.4 demonstrates that the polymer, in small concentrations, is more effective in protecting the enzyme than the nucleoside diphosphate at similar concentrations, suggesting that the relative protective effect of the substrates is related to the affinity of the enzyme for the substrate. Much more nucleoside diphosphate is required to saturate the enzyme than polymer (Grunberg-Manago, 1963).

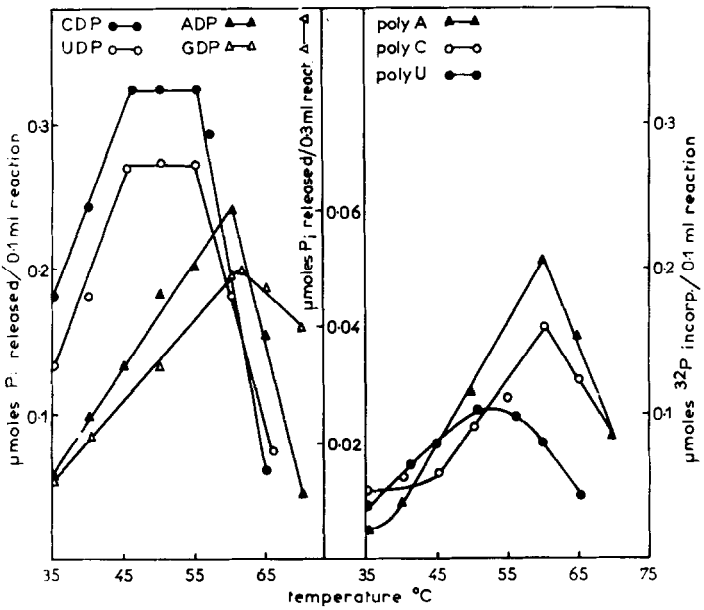

Fig.3 Effect of Temperature on the Rates of Polymerization and Phosphorolysis.

The reaction mixture (0.1 ml) for ADP, UDP and CDP polymerization contained 15 μg/ml of enzyme and the following in mM concentrations : tris buffer, pH 8.2, 100; EDTA, 1.0; $MgCl_2$, 9.0; and ADP, UDP or CDP, 20. For GDP polymerization, the mixture (0.3 ml) contained 75 μg/ml of enzyme, 2 mM GDP, 0.6 mM $MgCl_2$, and 150 mM tris buffer, pH 8.2. The incubation period for UDP and GDP polymerization was 30 minutes, for CDP, 20 minutes, and for ADP, 10 minutes. Because of the different times of incubation, the curves are not strictly comparable and show only optimal temperatures of reaction for each nucleoside diphosphate. For the phosphorolysis of poly A, poly U, and poly C, the reaction mixture (0.1 ml) contained 28 μg/ml of enzyme and the following in mM concentrations : tris buffer, pH 8.2, 150; $MgCl_2$, 7.5; EDTA, 0.5; poly A, poly U, or poly C, 3.4; and P_i^{32}, 10 (S.A., 1.5×10^6 cpm/μmole The incubation time was 3 minutes.

Protection by the nucleoside diphosphate does not appear to be a result of contamination by polymer formed during the preincubation period. When an excess of EDTA is added along with the nucleoside diphosphate to complex any metals added with the substrate, the same degree of protection is observed. Furthermore, there is no detectable release of inorganic phosphate or incorporation of C^{14}-ADP into polymer during the preincubation time, indicating the absence of polymerization.

All four nucleoside diphosphates and the corresponding homopolymers protect to some extent against heat inactivation. The effect, moreover, is specific for the substrates of the enzyme; nucleoside monophosphates, nucleoside triphosphates, polyvinylsulfate and calf thymus DNA are all ineffective. Furthermore, as shown in Fig.4, oligonucleotides with free 3'-OH terminal groups

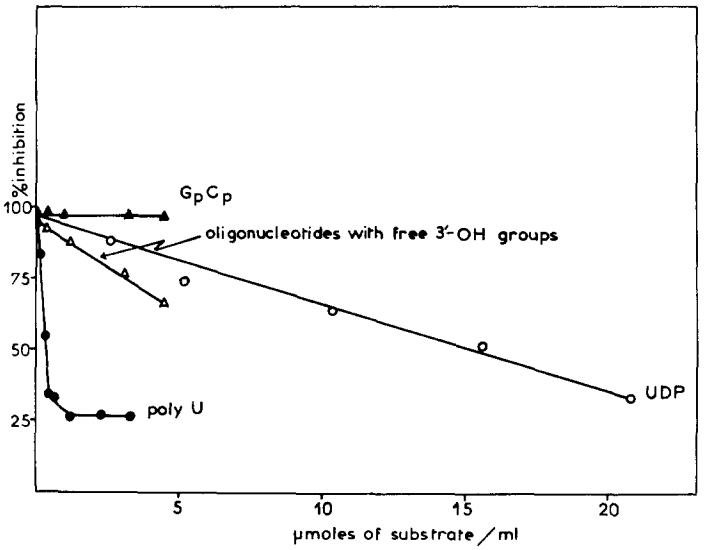

Fig.4 Protection of Polynucleotide Phosphorylase against Heat Inactivation by Various Substances.

The experimental conditions were the same as for Fig.1, except that the mixtures contained 1.0 μg of enzyme, and poly U, UDP, 3'-OH oligonucleotides or GpCp in the concentrations indicated in the figure. After preincubation, 0.9 μmoles $MgCl_2$, and UDP, poly U, 3'-OH oligonucleotides or GpCp were added in amounts such that for each series the final concentration of these substances was constant. The percentage inhibition is based on a control which was not preincubated.

protect the enzyme, whereas those with an esterified 3'-OH end do not. It is interesting that, while oligonucleotides ending with either a 3'-OH group or a 3'-phosphate group eliminate the lag phase in Azotobacter agilis, only those with a free terminal 3'-OH are incorporated into the newly formed polymer (Singer et al., 1957 and 1960). Both oligonucleotide preparations used in these experiments were able to overcome the lag phase produced by treatment of the enzyme with urea (Williams and Grunberg-Manago, 1964).

The phenomenon of protection against thermal inactivation by substrate or product, which has been observed with many other enzymes (Lumry, 1959), may be useful in studying the nature of the active site of polynucleotide phosphorylase. If, as appears to be the case, the enzyme is protected only by those substances which

can interact with the active site, then one might be able to distinguish between the groups on the substrate necessary for its attachment to the enzyme and those essential for the formation of reaction products with suitable substrate analogues, such as arabinosyl-CDP or deoxy-CDP (Cardeilhac and Cohen, 1964). Such studies are now in progress in this laboratory.

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