Polynucleotide Phosphorylase of Micrococcus luteus

STUDIES ON THE POLYMERIZATION REACTION CATALYZED BY PRIMER-DEPENDENT AND PRIMER-INDEPENDENT ENZYMES

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SUMMARY

Primer-dependent polynucleotide phosphorylase catalyzes the polymerization of ADP even in the absence of oligonucleotide primer. The reaction is very slow compared to the rate in the presence of primer, but the extent of reaction is directly proportional to time. Autocatalytic kinetics and long lags, observed earlier with cruder enzyme preparations, are absent with highly purified enzyme. The products of the reaction are very long polymer chains and do not serve as primers. No short chain intermediates are detectable and polymerization is therefore processive. Polymerization by primer-independent enzyme is also processive. In unprimed synthesis the apparent Km for ADP with dependent enzyme is several orders of magnitude higher than that found with independent enzyme.

In the presence of oligonucleotide primer, dependent enzyme catalyzes the addition of mononucleotide residues to the primer. The reaction is not processive; no very long chain polymer is made and short chain intermediates are readily detected. On the other hand, primer-independent enzyme does not incorporate primer, if supplied, into long chain polymer; under these conditions polymer synthesis de novo continues in a processive fashion.

Polynucleotide phosphorylase catalyzes the reversible synthesis of polynucleotides from ribonucleoside diphosphates, with the release of inorganic phosphate. Early studies demonstrated that with enzyme preparations from Micrococcus luteus (formerly Micrococcus lysodeikticus), the polymerization depended upon the addition of short (n > 2) polynucleotides with unesterified C-3'-hydroxyl groups (1–3). The added oligonucleotide served as a primer and was incorporated into the resulting polymer as the 5' terminus.

More recently, polynucleotide phosphorylase of M. luteus was also obtained in a primer-independent form, referred to as Form-I (4–7). This enzyme may be converted into a primer-dependent form (Form-T) by limited proteolysis with trypsin (4, 8–10). Form-T is reconverted to a new primer-independent form by reduction with sulfhydryl reagents (6). Form-T is easily distinguishable from Form-T on polyacrylamide disc electrophoresis while reduced Form-T has the same mobility as Form-T itself (6). The electrophoretic mobility of Form-T is identical with that of the early preparations that were primer dependent, as isolated (4).

Neither the primer independence of Form-I nor the primer dependence of Form-T is absolute. Polymerization of ADP by Form-I may be stimulated up to 2-fold by oligonucleotide (6), while Form-T does catalyze polymerization in the absence of primer, albeit with difficulty.

Earlier studies on the kinetics of polymerization by primer-dependent M. luteus enzyme, in the absence of oligonucleotides, indicated that a lag period occurred during which little or no reaction could be detected (3, 8, 9). After the lag period polymerization followed one of two courses. In some cases the reaction proceeded in a linear fashion (3) and in others it appeared to proceed at a constantly accelerating rate (8). The significance of these variations is unknown, given the fact that most of the enzyme preparations were relatively crude. Nevertheless the results suggested that primer-dependent enzyme could initiate polymer synthesis only with difficulty. It was generally believed that the lag represented a period during which the formation of polynucleotides occurred slowly and that the resulting chains could then act as primers, thereby leading to an increased reaction rate. The sometimes noted autocatalytic kinetics were consistent with such an interpretation.

These findings were in contrast to those obtained with enzyme from other bacterial sources, which, in general, did not require primer except in special circumstances. For example, highly purified Escherichia coli polynucleotide phosphorylase was not

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1 The Eighth Edition of the Catalogue of the American Type Culture Collection (1968) indicates that strain number 4698, formerly classified as Micrococcus lysodeikticus, is now reclassified as Micrococcus luteus. The enzyme described in this paper was purified from cells obtained commercially (Miles Laboratories, Inc.). The Miles Laboratories' catalogue designation is M. lysodeikticus (ATCC 4698). Therefore, we now refer to these cells as M. luteus.

2 Form-I is a primer-independent M. luteus (formerly M. lysodeikticus) polynucleotide phosphorylase. Form-T is derived from Form-I by limited tryptic hydrolysis.
dependent on primer (11) but a lag phase, that could be eliminated by oligonucleotides, was induced by a variety of treatments (11, 12). In addition, some preparations of enzyme from Azotobacter agilis which could not later be reproduced, were greatly stimulated by oligonucleotide primer (13). These data have been reviewed in detail (14).

Using highly purified Form-T from M. luteus, we have now re-examined the kinetics of polymerization by primer-dependent Form-T enzyme, and the results are reported here.

**Experimental Procedure**

**Nucleotides**—ApApApA was obtained from Miles Laboratories. *H*-pApApA was prepared by degrading 150 µg of *H*-poly A with sheep kidney endonuclease according to the method of Kasai and Grunberg-Manago (15). The digest, which contains primarily (pA)* with n from 2 to 6, was deproteinized repeatedly with chloroform and isoamyl alcohol (16). Deproteinization with phenol was avoided because of the solubility of oligonucleotides in phenol. In the chloroform-isoamyl alcohol procedure less than 1% of the oligonucleotide is soluble in the organic phase.

The solution of oligonucleotides was applied to a column (2.5 × 38 cm) of microgranular diethylaminoethyl cellulose (DE-52, Whatman) prepared as described by Tener (17) and equilibrated with 0.05 M NaCl containing 0.01 M Tris-HCl, pH 7.5. The elution of oligonucleotides was carried out with a linear gradient (2 liters total) from 0.05 to 0.3 M NaCl in the presence of 7 M urea and 0.01 M Tris-HCl, pH 7.5 (17). Appropriate fractions were pooled and desalted by a modification of the method of Rushizky and Sober (18) in which ammonium bicarbonate was replaced by triethylammonium bicarbonate, pH 7.9. The purity and chain length of the oligonucleotides was determined by paper chromatography with known markers. Although the peaks on the column were symmetrical and well separated, significant contamination with oligonucleotides of both lower and higher chain length was detected.

N-Ethylmaleimide, ADP, CDP, UDP, and poly A were obtained from Schwarz BioResearch, as was *32P*-ADP. Tripsin, twice crystallized, was from Worthington. Gel electrophoresis materials were from Canal Industrial Corporation, Bethesda, Maryland.

**Enzyme**—Polynucleotide phosphorylase was prepared from M. luteus through the hydroxylapatite step, as previously described (5, 7). The primer-independent enzyme (Form-I) had a specific activity of 53 phosphorylase units per mg of protein (1 phosphorylase unit is the amount of enzyme catalyzing the formation of 1 µmole of ADP from poly A in 15 min) and 193 polymerization units per mg (1 polymerization unit catalyzes the incorporation of 1 µmole of ADP into poly A in 15 min). Protein was calculated from the absorbance at 280 µm, with the extinction coefficient reported by Klee (7). Two preparations of primer-dependent enzyme (Form-T) were used and both gave identical results. One (Preparation A) was prepared by limited proteolysis with trypsin, from the Form-I enzyme just described (7). The second preparation of Form-T (Preparation B) was prepared by limited proteolysis of a crude enzyme preparation with trypsin (8). Although the latter material was then purified through the hydroxylapatite step, it did not attain the purity usually found at that stage. The specific activity was 12.3 phosphorylase units per mg of protein.

Form-T enzyme was alkylated with N-ethylmaleimide as described previously (7).

Sheep kidney nuclease was prepared according to the method of Kasai and Grunberg-Manago (15).

**Enzyme Assays**—Polymerization was routinely assayed by measuring the release of Pi from ADP, or other nucleoside diphosphates. When piimer was used it was ApApApA unless otherwise noted. The reaction mixtures contained 0.02 M nucleoside diphosphate, 0.4 mM EDTA, 5 mM MgCl₂, 0.1 M Tris-HCl, pH 6.0, 0.9 mg per ml of dialyzed bovine serum albumin, and enzyme. Incubations were all at 37°.

At the end of the incubation period, the reaction was stopped by the addition of 20 volumes of ice-cold 2.5% perchloric acid. After 10 min in ice, the samples were centrifuged and the supernatant fluid used to assay for Pi by the method of Fiske and SubbaRow (19). When the incorporation of labeled substrates into polymer was measured, the acid-precipitable material was collected on Whatman GF/C glass filters, 2.4 cm, and washed three times with 3 ml of ice water, dried, and counted in toluene scintillator.

The general procedure for a time course was to assemble the reagents and begin the reactions with the addition of enzyme. At the various time points, 0.05-ml samples were withdrawn and added to 0.95 ml of 2.5% perchloric acid. The zero time aliquot, which was removed immediately after the addition of enzyme, served as the blank.

**Disc Gel Electrophoresis**—Disc gel electrophoresis was used to check the purity of polynucleotide phosphorylase and to distinguish Form-I from Form-T. The procedures have been described (4, 7).

For analysis of poly A product, disc gel electrophoresis was carried out with 5% acrylamide gels according to the methods of McPhie, Hounsell, and Gratzer (20) and Gould (21) except that samples were applied in a sample gel at pH 8.0. For a period of 35 min, 5 µa per tube were applied at room temperature. The current was stopped when the bromophenol blue marker was still about 1 cm from the bottom of the tube. After completion of the run the gels were sliced into about 40 segments with an "egg slicer" (22) and the slices were placed into glass scintillation vials. Thirty percent hydrogen peroxide (75 µl) was added, the vials were tightly capped, and then heated for 8 to 12 hours at 55° to dissolve the gel (23). At the end of that period, the vials were cooled, and 1.0 ml of water was added to each. After shaking, 10 ml of Triton-toluene scintillator (24) was added to each vial and the samples were counted.

As expected from the data of McPhie et al. (20) preliminary experiments established that polymers of chain length 700 or greater, migrate 1 cm or less into the separating gel under the conditions used. Polymers of chain length 730 and 1200, kindly provided by Dr. Gary Felsenfeld (25), were used to calibrate the gels. Some of the polymer remains in the stacking gel. ADP and oligonucleotides (n ≤ 6) migrate in front of the dye marker while RNA is found just behind the dye marker. Free enzyme (Form-I) migrates about 27% of the length of the gel; in a gel yielding 40 slices this corresponds to approximately Fraction 11. Slicing of the gels into segments did not afford reliably uniform yields of slices. Slices, thus accounting for the variation in the number of slices obtained from each gel as well as for some of the irregularities in the pattern of radioactivity. In the figures, the numbering of slices starts from the top of the gel and the first 3 to 5 slices, in general, represent stacking gel. The large peak of radioactivity at the bottom of the gel is mainly ADP.

**Paper Chromatography**—Certain reaction mixtures were...
Fig. 1. Polymerization of ADP by Form-T enzyme. Polymerization was measured by the standard assay described under "Experimental Procedure." In A, the concentration of Form-T enzyme (Preparation A) was 96 µg per ml. In B, the concentration was 19 µg per ml and (Ap)₃A was 1 mM when added. At the indicated times, 50-µl samples were removed and used to determine the release of Pi. The ordinate gives the nanomoles of Pi produced per 50-µl sample. In B, 1, with 0.25 mM (Ap)₃A; 0, without (Ap)₃A.

Fig. 2. Polymerization of ADP by varying amounts of Form-T enzyme. Polymerization was measured by the standard assay as described under "Experimental Procedure." In A, the numbers on the graph represent the microliters of Form-T enzyme (Preparation B) present in 50 µl of reaction mixture. This enzyme had a protein concentration of 3.2 mg per ml. At the indicated times, 50-µl samples of the incubation mixture were withdrawn. The results are expressed as nanomoles of Pi released per 50-µl aliquot of reaction mixture. B shows the rate of polymerization (as determined from A) as a function of the concentration of enzyme. The rate is expressed as nanomoles of Pi released per 50 µl of reaction mixture per min.

Fig. 3. Polymerization of ADP by Form-T enzyme in the presence and absence of (Ap)₃A. Polymerization was measured by the release of Pi, and by incorporation of radioactivity from ³H-ADP into acid-precipitable material as described under "Experimental Procedure." The concentration of Form-T enzyme (Preparation B) was 80 µg per ml. At the indicated times, 50 µl samples were removed. The specific activity of the ADP was 1.5 × 10⁶ cpm per µmole. 1, 0.25 mM (Ap)₃A, Pi release; A, 0.25 mM (Ap)₃A, ³H-ADP incorporation; 0, no (Ap)₃A, Pi release; 0, no (Ap)₃A, ³H-ADP incorporation.

Fig. 4. Polymerization of ADP by N-ethylmaleimide-treated Form-T enzyme. Polymerization was measured by the standard assay described under "Experimental Procedure." The concentration of (Ap)₃A was 0.25 mM where indicated. The concentration of N-ethylmaleimide-treated enzyme (Preparation B) was approximately 41 µg per ml. 50-µl samples were removed at the indicated times and the ordinate gives the nanomoles of Pi released per 50 µl. 1, with (Ap)₃A; 0, no (Ap)₃A.

RESULTS

Polymerization by Form-T Enzyme

Kinetics of Polymerisation by Form-T—The polymerization of ADP by Form-T in the absence of primer is shown in Fig. 1A as a function of time. The reaction proceeds at a linear rate from zero time, as measured by release of Pi. The primer dependence of this enzyme preparation is demonstrated in Fig. 1B, which compares the rate of polymerization in the presence and absence of ApApApA. The concentration of enzyme in Fig. 1B is 0.2 that in Fig. 1A. The preparation is stimulated 20-fold by the presence of 0.20 mM (Ap)₃A. Identical results were obtained with both preparations of Form-T as described under "Experimental Procedure."

The rates of polymerization of ADP by Form-T at several concentrations of enzyme are shown in Fig. 2A. In every case, the extent of reaction is proportional to the time. The rates of polymerization observed in Fig. 2A are plotted as a function of enzyme concentration in Fig. 2B. The rate is directly proportional to the amount of enzyme. Therefore, dependence on primer is not overcome by increasing the enzyme concentration.

Similar kinetics were obtained when the course of the reaction was followed by measuring the formation of acid-insoluble poly A product. In the absence of primer the amount of poly A

* The concentration of ApApApA is given as concentration of oligonucleotide, not as monomer units.
large amounts of Form-T on polyacrylamide gels indicated slight activity of Form-T is clearly discernible when gels are incubated cannot be ascribed to contamination with reduced Form-T.

It is necessary to consider the possibility that the polymerization by Form-T enzyme in the absence of primer might actually reflect contamination of the enzyme with either Form-I or reduced Form-T. However, several observations indicate that Form-T is indeed able to catalyze polymerization in the absence of primer at a linear, albeit slow, rate.

To eliminate the problem of contamination by reduced Form-T, a sample of Form-T enzyme was first treated with β-mercaptoethanol and then with N-ethylmaleimide as previously described (6, 7). The reduction converts Form-T to a primer-independent form; reaction with N-ethylmaleimide alkylates the reduced enzyme and reconverts it irreversibly to a primer-dependent state. The treatment results in some loss of over-all activity (6, 7). The alkylated enzyme does, however, catalyze the polymerization of ADP; the reaction is linear with time and the rate is stimulated about 10-fold by addition of ApApApA (Fig. 4). Thus, the linear rate of polymerization by Form-T cannot be ascribed to contamination with reduced Form-T.

The possibility that the reaction results from contamination of Form-T by Form-I also appears unlikely. Electrophoresis of large amounts of Form-T on polyacrylamide gels indicated slight activity in the region characteristic of Form-I. However, the activity of Form-T is clearly discernible when gels are incubated with ADP and the resulting polymer stained with acridine orange (4). In this situation the two enzyme forms are widely separated on the gel.

The experiments shown in Figs. 5 and 6 support the conclusion that Form-T has inherent polymerizing activity, and in addition they reveal an important difference between primer-independent and primer-dependent enzyme. The polymerization of ADP by Form-I (Fig. 5) and by Form-T (Fig. 6) was studied as a function of ADP concentration, and in the experiments the enzyme concentration was 30 μg per ml and the incubation time 150 min.

The velocity is expressed as nanomoles of Pi released per min per ml of stock enzyme solution (6.2 mg of protein per ml).

The reaction may reflect the redistribution of chain sizes as the reaction approaches equilibrium (26), as well as the relatively short chain product produced under these conditions (see below).

The Michaelis constants of Form-I and Form-T, in the absence of primer, differ by several orders of magnitude. In the presence of primer, the apparent Kₘ concentrations and approaches that found for Form-I in either the presence or absence of primer. In addition, the apparent Kₘ for reduced Form-T (Fig. 6) is similar to that obtained with Form-I.

It should be stressed that the apparent Kₘ values are presented here only for comparison, and do not represent true kinetic constants. Nevertheless they indicate that the linear rate of

The stated concentrations of ADP are the total ADP concentrations and not corrected for the ADP that is complexed with Mg²⁺. The substrate for polymerization appears to be free ADP, not the complex (the evidence for this is summarized in Reference 27).
polymerization by Form-T in the absence of primer cannot result from contamination either with Form-I or reduced Form-T.

Substrate Specificity—In view of reports (9, 10) that trypsin-treated polynucleotide phosphorylase demonstrates marked substrate specificity in polymerization, the polymerization UDP and CDP was tested with Form-T. Both substrates yielded linear time courses in the absence of primer. Under the conditions used, the relative activities toward ADP, UDP, and CDP were 1.1, 0.9, and 0.26 nmoles of P, released per min per μl of enzyme solution, respectively. The rates of polymerization of UDP and CDP were stimulated 10- to 20-fold by the addition of primer. Although Form-T enzyme prepared in this laboratory by trypsin degradation and characterized by electrophoretic mobility, primer dependence, and conversion to primer independence by treatment with sulfhydryl, has this property, it is possible to obtain enzyme with properties similar to those described by Fitt et al. (9, 10). With one trypsin-treated preparation obtained from Dr. James Willis of P-L Biochemicals, the ratio of activity with primer to that without primer was 2.2 with ADP, 17 with UDP, and 15 with CDP. Treatment with sulfhydryl reagents did not stimulate activity in the absence of primer with any of the nucleoside diphosphates. On disc gel electrophoresis there was a single activity band with a mobility close to, but not identical with, that of Form-T. At present we are not certain of the cause of the alternate results of trypsin digestion, but believe it to be related to differences in the Form-I preparations used. We are grateful for the cooperation of Dr. Willis in this, and other matters.

The experiments just described show that in contrast to less highly purified enzyme, the recent preparations of dependent enzyme show neither a lag nor any sort of autacatalytic kinetics in the polymerization reaction. The earlier demonstrations of lags, or autacatalytic curves, were interpreted to indicate that some product of the reaction, presumably an oligonucleotide, was serving to stimulate the rate of polymerization. We therefore studied the nature of the polymer products with the various enzyme forms.

Effect of Reaction Products on Rate of Polymerization by Form-T Enzyme—Using the highly purified Form-T, we investigated directly the possibility that stimulatory material is formed during the polymerization reaction. A standard reaction mixture was set up containing Form-T enzyme and no primer. Samples were withdrawn at intervals and heated at 75° for 10 min to inactivate the enzyme. (Preliminary experiments demonstrated that this treatment completely destroys polynucleotide phosphorylase activity but does not affect the activity of oligonucleotide primer.) The samples were then cooled rapidly, and an amount of fresh enzyme equal to that originally present was added, and incubation at 37° was resumed. The time course of the reaction was followed by P_i release in the usual manner both in the original mixture (Fig. 7, main time course) and in the treated samples (Fig. 7, A, B, and C). One portion of the original incubation mixture was heated at zero time as a control (Fig. 7, A, B, and C). The samples in Fig. 7, A, B, and C, were removed at the indicated times, when 2.2 and 67 nmoles of ADP had been polymerized per 50 μl of reaction mixture, respectively. After the addition of fresh enzyme the rate of polymerization was, in each case, about 50% greater than in the starting mixture. This experiment suggests that little or no stimulatory oligonucleotide

![Fig. 7. Effect of reaction products on the rate of polymerization by Form-T enzyme.](http://www.jbc.org/)

![Fig. 8. The effect of sheep kidney endonuclease on the kinetics of ADP polymerization by Form-T enzyme in the absence of oligonucleotide primer.](http://www.jbc.org/)

The ordinate gives the nanomoles of P_i released per 50 μl of reaction mixture, in the main time course, A, B, and C, respectively. The zero time value was subtracted from the later measurements in each instance. The ordinate gives the nanomoles of P_i per 50 μl of reaction mixture, in the main time course, A, B, and C, respectively.

![Graph showing the effect of reaction products on the rate of polymerization by Form-T enzyme.](http://www.jbc.org/)

![Graph showing the effect of sheep kidney endonuclease on the kinetics of ADP polymerization by Form-T enzyme in the absence of oligonucleotide primer.](http://www.jbc.org/)
is produced during the polymerization reaction, a finding that is confirmed by experiments described below. Thus, for example, when 67 nmoles of ADP are polymerized per 50 $\mu$L of reaction mixture, the concentration of polymer, in AM residues, is 1.34 $\mu$m per ml. If this were composed entirely of tetranucleotide, it would represent a concentration of 0.34 nM. Fig. 1B shows that at such a concentration, polymerization should be stimulated approximately 20-fold. The autocatalytic curves obtained in work with crude enzymes could have resulted from the secondary destruction of the polymer product into oligonucleotides by a contaminating endonuclease. As shown in Fig. 8, addition of endonuclease to reaction mixtures containing ADP and Form-T enzyme does indeed lead to autocatalytic curves. The sheep kidney endonuclease used is known to generate oligonucleotides with unesterified terminal C-3'-hydroxyl groups, and phosphomonoesterified terminal C-5'-hydroxyl groups (i.e. (PA)$_n$ (15)).

Size of Poly A Products—Direct analysis of the chain length of the polymer product was carried out by means of electrophoresis on polyacrylamide gels and paper chromatography. The former procedure was used to detect poly A of chain length greater than 50 residues and the latter to study the formation of oligonucleotides, $n \leq 9$. In the electrophoresis experiments, which will be presented first, $^{14}$C-ADP was used as substrate and duplicate samples of the reaction mixtures were removed at various times. One sample was used to measure the $P_i$ formed in the standard way. The other sample was added to EDTA to stop the reaction and the mixture was applied to the top of a 5% polyacrylamide gel. After electrophoresis the gels were sliced and the radioactivity in each segment was determined as described under “Experimental Procedure.”

The main purpose of these experiments was to study the products accumulated with Form-T enzyme, but control experiments with Form-I were also carried out. Fig. 9 shows the results obtained upon gel electrophoresis of the products of polymerization with Form-I in the absence and in the presence of (Ap)$_n$A. In both cases, very high molecular weight poly A (top of the gel) is detectable even after very little total reaction. The first times shown on Fig. 9 represent 3% utilization of the available ADP in the experiment with no primer, and 7.5% when ApApApA was present. As the reaction proceeds, more and more large poly A accumulates. Calibration of the gels with poly A of known size (see “Experimental Procedure”) indicates that the product has a chain length of 700 or greater. It is also notable that relatively few counts were detected between the peak of ADP substrate (bottom of the gel) and the peak of large poly A. The radioactivity in this region appears to increase some with time and may result from a redistribution of chain length as the reaction proceeds (see discussion of Fig. 3 above) or a minor nucleic acid contamination.

Results of a similar experiment with Form-T enzyme are shown in Fig. 10. In the absence of (Ap)$_n$A, high molecular weight poly A is apparent at the earliest time studied, and as with Form-I (Fig. 9) the amount of polymer increases with increasing release of $P_i$. Again, only small amounts of labeled polymer are apparent between the ADP and the material at the top of the gel. The result with Form-T in the presence of (Ap)$_n$A is strikingly different (Fig. 10). At none of the points studied was any poly A detected at the top of the gel. With time, the amount of poly A with mobility less than that of tRNA increases.

Fig. 9. Polyacrylamide gel electrophoresis of the products of the polymerization of ADP by Form-I enzyme. The results were as described under “Experimental Procedure.” The specific radioactivity of the ADP was $2 \times 10^6$ cpm per mole. Form I concentration was 10 $\mu$g per ml in the experiment in the absence of oligonucleotide and 14 $\mu$g per ml when (Ap)$_n$A (0.25 mM) was present. Incubation was at 37°C. At various times, 50 $\mu$L samples were withdrawn for determination of $P_i$ release; and a time course was plotted. From this curve the nanomoles of $P_i$ formed per min per slice was determined (numbers at the upper left hand corners of the graphs). The samples (50 $\mu$L) withdrawn for electrophoresis were added to 1.2 pmolcs of $^{32}$P-ADP to stop the reaction. They were then mixed with 0.2 ml of unpolymerized stacking gel mixture, added to the top of the previously formed gel columns and allowed to polymerize under a fluorescent light before current was applied. The details of the electrophoretic technique, the slicing, and the counting of the gel are described under “Experimental Procedure.” The ordinate gives the counts per min per slice.

Fig. 10. Polyacrylamide gel electrophoresis of the products of the polymerization of ADP by Form-T enzyme. The experiments were as described for Fig. 9. The enzyme concentrations (Form-T. Preparation B) were 64 $\mu$g per ml in the absence of (Ap)$_n$A and 32 $\mu$g per ml in the presence of (Ap)$_n$A.
the short chain products of polymerization. Form-I, even in the presence of primer, synthesizes long chain in the presence of oligonucleotide primer. The observation that the final concentration of enzyme was 32 μg per ml. The enzyme was then diluted 10-fold with 1 mg per ml of bovine serum albumin and added to the reaction mixture. The final concentration of enzyme was 32 μg per ml.

somewhat, and is apparent as a low level of radioactivity along the whole length of the gel. When Form-T enzyme is first reduced with mercaptoethanol to convert it to a primer-independent form (6), the appearance of high molecular weight poly A (top of the gel) is again detected early in the reaction (Fig. 11).

Thus, the experiments in Figs. 9 through 11 indicate that long chain poly A (n > 700) is synthesized very early during the polymerization reaction, in all cases except with Form-T enzyme in the presence of oligonucleotide primer. The observation that Form-I, even in the presence of primer, synthesizes long chain material was of interest and led us to investigate the nature of the short chain products of polymerization.

The incubations used were similar to those just described, except that either the ADP or the oligonucleotide primer was labeled. Samples of the reaction mixtures were removed at various times and used to determine the release of Pi, or the nature of the accumulated oligonucleotides (by chromatography on DEAE paper). With Form-I enzyme, 3H-pApApA disappears and the radioactivity is converted to longer oligonucleotides (Fig. 12). At the first time point, the main product is tetranucleotide, but as the reaction proceeds, longer labeled oligonucleotides appear. Very little, if any, of the oligonucleotide ever appears at the origin where long chain poly A is found. Therefore, while long polymer is being synthesized by Form-I in the presence of oligonucleotide (see Fig. 9), little if any of the polymer forms by extension of oligonucleotide primer. An experiment (not shown) identical with that shown in Fig. 12 for Form-I, but with labeled ADP and unlabeled oligonucleotide, showed the accumulation of labeled polymeric material at the origin, with a suggestion of peaks corresponding to the expected labeled tetra- and pentanucleotides. In the absence of oligonucleotide, no counts at all appeared on the chromatogram between the ADP and the origin.

Fig. 12 also shows the results of an experiment with Form-T enzyme and 3H-pApApA. In this case, too, the trinucleotide is utilized and longer oligonucleotides appear; however, the label is incorporated into the material at the origin, indicating that the polymer is synthesized by extension of the oligonucleotide chains. Furthermore, the rate of 3H-pApApA utilization relative to P1 release is much higher for Form-T than for Form-I. When the same experiment (not shown) was repeated with unlabeled trinucleotide and radioactive ADP, the oligonucleotides between pApApA and the origin were labeled and the pattern of label was consistent with that shown for Form-T in Fig. 12. In the absence of oligonucleotide (unprimed synthesis by Form-T) no counts at all appeared between ADP and the origin. The specific activity of the ADP was such that intermediate oligonucleotides at a level 0.05% of the polymer synthesized would have been easily detected. Therefore unprimed synthesis by either Form-I or Form-T does not lead to the accumulation of detectable oligonucleotide intermediates.

In the course of carrying out these experiments on the incorporation of 3H-pApApA into polymer, the formation of acid insoluble radioactivity was measured as a function of time. With...
Form-T enzyme, the rate of incorporation of \( ^{3}P\)-pApApA was essentially constant and there was approximately 1 \( \mu \)mole of \( ^{3}P\)-pApApA incorporated per 40 \( \mu \)moles of P\(_{1}\) released. With Form-I enzyme, an apparent incorporation of \( ^{3}P\)-pApApA into acid-insoluble material was also noted; 1 \( \mu \)mole of \( ^{3}P\)-pApApA per 140 \( \mu \)moles of P\(_{1}\) released. However, a control experiment showed that under these conditions, there is coprecipitation of \( ^{3}P\)-pApApA and poly A; the extent of this coprecipitation depends on the amount of poly A and accounts in large measure for the observed incorporation with Form-I. With the value of 1 \( \mu \)mole of \( ^{3}P\)-pApApA per 140 \( \mu \)moles of P\(_{1}\) released as the value for coprecipitation, the results with Form-T can be suitably corrected. This calculation indicates that 1 \( \mu \)mole of \( ^{3}P\)-pApApA is incorporated per 56 \( \mu \)moles of P\(_{1}\) released, a chain length consistent with the electrophoretic and chromatographic findings.

**DISCUSSION**

The evidence presented here shows that primer-dependent polynucleotide phosphorylase from *M. luteus* (Form-T) does indeed catalyze polymerization in the absence of oligonucleotide primer albeit at a very slow rate. In distinction to earlier results (3, 8, 9, 13) the rate of the reaction appears to be linear with time and is directly proportional to the enzyme concentration. The autacatalytic kinetics and apparent long lag periods observed earlier were probably artifacts arising from contamination of the enzyme preparations with nucleases.

Polyribonucleotides which can prime the polymerization of ADP by Form-T are apparently not produced in significant amount during unprimed synthesis. This conclusion is supported by several observations in addition to the linear polymerization rate. (a) In the experiment in which fresh enzyme was added to partly reacted and heated reaction mixtures, only a 50\% increase over the expected rate was observed. Significant accumulation of primer would have resulted in substantially greater stimulation. The small increase may result from an undetectable amount of oligonucleotide present in the reaction mixtures or produced by degradation of polymer during heating. It may also represent protection of the enzyme against heat inactivation by the components of a partly reacted mixture. (b) Direct investigation of the size of the polymer produced during unprimed synthesis by Form-T demonstrated that no short chain length material is produced to any significant extent. No material of intermediate chain length was detected either on gel electrophoresis (which would reveal rather high molecular weight intermediates) or on ion exchange paper chromatography (which would reveal small oligonucleotide intermediates). Poly A of chain length greater than 700 is detectable after a small percentage of the ADP has reacted, and as polymerization proceeds, the amount of this material increases. Priming by this product is not expected since it is known that long chain poly A does not serve as a primer for primer-dependent *M. luteus* enzyme (12, 28, 29).

Thus, unprimed synthesis by form-T enzyme appears to be processive (24); long chain polymers are synthesized one at a time without the accumulation of short chain intermediates. Polymerization by primer-independent polynucleotide phosphorylase is also processive, as indicated previously in experiments with *E. coli* enzyme, and demonstrated here for *M. luteus* enzyme. The synthesis of one polymer chain at a time is consistent with the fact that in the reverse reaction phosphorylisis of long chain polymers by both Form-I (5) and Form-T (27), and *E. coli* enzyme (30) is also processive; the enzyme phosphorylates a given polynucleotide to completion before initiating phosphorylation of another chain.

In the experiments summarized thus far for unprimed polymerization of ADP by Form-T, the reaction has the same properties as synthesis by Form-I or reduced Form-T. The data indicate, however, that the unprimed synthesis by Form-T cannot be ascribed to contamination of the preparation with Form-I or reduced Form-T. The most striking observation in this regard is the difference in the kinetics of polymerization as a function of ADP concentration between Form-T on one hand, and Form-I or reduced Form-T on the other. The apparent difference in the concentration of ADP required for half the maximal rate is at least two orders of magnitude. The difference in the apparent \( K_{m} \) value may or may not be related to lower binding affinity of Form-T for ADP. Kinetic data obtained with the reverse reaction, namely, phosphorylation of oligonucleotides, suggest that the ADP binding site is unaltered. Thus, the \( K_{m} \) for P\(_{1}\) is the same with Form-I and Form-T, the \( K_{m} \) values for dADP are identical with the two enzyme forms, and the \( K_{m} \) values for adenosine 5'-methylene diphosphate are very similar (27). However, these data were of course obtained in the presence of oligonucleotide. Since oligonucleotides do alter the apparent \( K_{m} \) for ADP in polymerization (see Fig. 6), it may be that in the phosphorylation experiments the presence of oligonucleotide similarly overcomes the inability of Form-T to bind nucleoside diphosphates. This effect may result from a conformational change in the structure of Form-T upon binding of oligonucleotide.

The formation of high molecular weight polymer by Form-T in the absence of primer is in marked contrast to the products of synthesis by the same enzyme in the presence of primer. In the latter case no poly A comparable in size to that produced in the absence of primer is detectable. Inspection of the polyacrylamide gels (Fig. 10) suggests that the bulk of the poly A formed is rather small, of the order of tRNA or less in size. Inspection of the paper chromatogram (Fig. 12) indicates that oligonucleotides of intermediate chain length accumulate during primed synthesis and are converted to the longer material in a nonprocessive way. This observation is consistent with the fact that phosphorylisis of oligonucleotides is also nonprocessive (27). Earlier, with cruder preparations of *M. luteus* enzyme, that were primer-dependent as isolated (1, 2, 4), Anderson (31) also observed that addition to oligonucleotide primers is non-processive. In contrast to the present findings, however, high molecular weight poly A (\( > 800 \mu \)m) equal to about 8 S material was also detected in this system. The explanation for this difference is unknown, but it is possible that the earlier enzyme preparation was a mixture of primer-independent and primer-dependent forms; incorporation of the oligonucleotide into 8 S material was not measured.

The evidence presented here indicates that with primer-independent Form-I, oligonucleotides are not incorporated into newly synthesized long poly A chains. The paper chromatograms show directly that none of the oligonucleotide ever appears at the origin of the chromatogram. A slow, nonprocessive incorporation of the trinucleotide pApApA into compounds a few units longer is observed and appears to represent a side reaction.
The preferred reaction in this instance is the processive synthesis of large polymer. Early experiments (32) also showed that oligonucleotide primer is extended in chain length with primer-independent enzyme. Although the point was not made explicitly at the time, the published data (32) show clearly that under such conditions the disappearance of pApApA primer in the presence of UDP can be largely accounted for by the production of (pAp)pU and (pAp)pU.

Although ADP polymerization by Form-I in the presence of oligonucleotide occurs essentially without primer utilization, oligonucleotide may stimulate the rate of P\textsubscript{i} release up to 2-fold (6). The mechanism of this stimulation would appear to be different from that operating with primer-dependent enzyme. It may be related to previous observations on the stimulatory effect of oligonucleotides of structure (Np), on primer-dependent A. agilis enzyme (32); because of the 3'-phosphate group such oligonucleotides cannot be incorporated into polymer and do not serve as primers for M. luteus Form-T (1, 2, 8).

The small stimulation of Form-I by oligonucleotides may also be related to recent observations of Godfrey, Cohn, and Grunberg-Manago (28). Very early in the polymerization of nucleoside diphosphates by primer-independent E. coli enzyme a lag phase is observed which can be overcome by very low concentrations of oligonucleotides as well as long chain poly A.

With both enzyme forms chain growth by addition of new nucleoside monophosphate residues to an added primer appears to be nonprocessive, in keeping with the nonprocessive phosphorolysis of oligonucleotides (27). This is the preferred reaction with Form-T, while synthesis de novo is preferred with Form-I, even when primer is supplied. A practical consequence of this finding is that Form-T is more suitable for the synthesis of primed synthesis but, are never detectable in synthesis de novo, that accounts for this. This conclusion is supported by the fact, that synthesis by extension of primer never becomes processive and the resulting chains never reach the length of those made de novo.

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