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Replication of poly dA and poly rA by a Drosophila DNA polymerase

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ABSTRACT

The activity of a 7.3S-8.3S Drosophila DNA polymerase was characterized in detail using poly dA•p(dT)$_{\overline{16}}$ and poly rA•p(dT)$_{\overline{16}}$. With poly dA•p(dT)$_{\overline{16}}$, Mg^{2+} ion was the preferred divalent cation, and enzyme activity was inhibited by K^{+} ion and by spermidine. With poly rA•p(dT)$_{\overline{16}}$, Mn^{2+} ion was the preferred divalent cation and enzyme activity was stimulated by K^{+} ion and by spermidine. The dependence of enzyme activity on the concentration of primer-template and on the ratio of primer to template was the same in both reactions. The two enzyme activities were identically inhibited by N-ethylmaleimide. Poly dA was replicated extensively and poly rA was replicated partially. The activation energy for poly dA replication was twice that for poly rA replication. Enzyme activity with poly dA•p(dT)$_{\overline{16}}$ was more stable to thermal inactivation than was enzyme activity with poly rA•p(dT)$_{\overline{16}}$. These studies suggest that the same enzyme responds to both the deoxy- and the ribohomopolymer template but that the mechanisms of replication may be different.

INTRODUCTION

The physical properties of vertebrate DNA polymerases and the levels of their activities with homopolymer primer-templates have been used to differentiate three DNA polymerases: α, β, and γ (1,2). Although the biological significance of DNA polymerase utilization of primed ribohomopolymer templates is uncertain, this activity has been characteristically associated with DNA polymerases-β and -γ, but not with DNA polymerase-α (1,2). For example, DNA polymerase-α is inactive with poly rA•p(dT)$_{\overline{16}}$, DNA polymerase-β is as active with poly rA•p(dT)$_{\overline{16}}$ as with activated DNA, and DNA polymerase-γ is more active with poly rA•p(dT)$_{\overline{16}}$ than with activated DNA (1,2). Although the DNA polymerases of eukaryotic microorganisms (3-7) and invertebrates (8-11) have properties in common with vertebrate polymerases, they may not fit simply into the three polymerase classes that have been defined for the vertebrate polymerases. This appears to be the case for three forms of DNA polymerase that have been isolated from Drosophila embryos (9,10).

Some of the physical and enzymatic properties of the three forms of Drosophila DNA polymerase suggest that they are related to DNA polymerase-α of vertebrate organisms: the enzymes eluted heterogeneously from gel filtration columns and DEAE-cellulose columns; they had sedimentation coefficients from 7.3S to 9.0S; and their DNA polymerase activities were inhibited by N-ethylmaleimide (MalNEt) and monovalent cations (10). However, other properties of the Drosophila enzymes suggest that they are related to DNA polymerases-β and -γ : the pH optimum for enzyme activity was pH 8.5; and two of the three forms of DNA polymerase, although physically similar to DNA polymerase-α, were able to synthesize poly dT in response to either poly dA or poly rA templates with oligo dT primers. We have studied these two DNA polymerase activities of the Drosophila DNA polymerase in greater detail, and the results of this study demonstrate that the details of the enzymatic activities are similar to those reported for DNA polymerase-γ (1,2,12) and for a Tetrahymena DNA polymerase (4). These studies establish a basis for correlating the structure and mechanism of action of the Drosophila DNA polymerase.

MATERIALS AND METHODS

Enzymes and Chemicals. DNA polymerase was purified from Drosophila melanogaster embryos as described in an earlier report (10). Either the Step 6 or Step 7 (7.3S-8.3S) DNA polymerases were used for these studies. The specific activities of the Step 6 and 7 enzymes, assayed with poly dA•p(dT)$_{\overline{16}}$ were about 15,000 units/mg of protein and 55,000 units/mg of protein, respectively.[1] The specific activities with poly rA•p(dT)$_{\overline{16}}$ were approximately three-fold lower (10). One unit of enzyme activity is defined as the amount of enzyme required to incorporate 1 nmol of dTMP into poly dT in 60 min at 35°C in the poly dA•p(dT)$_{\overline{16}}$ reaction mixture. Chemicals and templates were described previously (10).

DNA Polymerase Assays. DNA polymerase activity was determined, as described previously (10), in 50 µl reaction mixtures that contained 50 mM Tris HCl (pH 8.5), 0.02% 2-mercaptoethanol, 0.3 mM dTTP, 40 µCi/ml [^{3}H]dTTP (40-50 Ci/mmol), 0.6 mg/ml bovine serum albumin (BSA), and either 5 mM $MgCl_2$ plus poly dA•p(dT)$_{\overline{16}}$ (0.1 mM dAMP, 0.01 mM dTMP) or 0.5 mM $MnCl_2$ plus

Footnote [1] -

We have recently become aware that the counting efficiency of [^{3}H]poly dT on DE-81 filters is four-fold greater than the counting efficiency of [^{3}H]dTTP (See also, reference 19). Therefore the specific activities are four-fold lower than we reported previously (10).

poly rA•p(dT)$_{\overline{16}}$ (0.1 mM rAMP, 0.01 mM dTMP). The K^+ concentration was 20 mM in standard poly dA•p(dT)$_{\overline{16}}$ reaction mixtures and was 80 mM in standard poly rA•p(dT)$_{\overline{16}}$ reaction mixtures (10). Specific changes in reaction conditions are indicated in the appropriate figure legends. [^{3}H]dTMP incorporation into poly dT was determined using DE-81 (Whatman) filter discs (10,13). The counting efficiency for [^{3}H]poly dT was about 20%, giving 60 cpm/pmol of dTMP incorporated (See footnote 1).

RESULTS

Divalent Cation Requirements for Enzyme Activity. DNA polymerase activity required divalent cations. A broad range of Mg^{2+} concentrations (2.5-10 mM) gave maximum activity with poly dA•p(dT)$_{\overline{16}}$ (Fig. 1A). Enzyme activity with poly dA•p(dT)$_{\overline{16}}$ in the presence of Mn^{2+} was maximum at 0.4 mM Mn^{2+}, but was only 17% of the maximum activity with Mg^{2+} (Fig. 1A). Mn^{2+} ion was the preferred divalent cation in poly rA•p(dT)$_{\overline{16}}$ reaction mixtures and the optimum Mn^{2+} concentration was 0.5 mM (Fig. 1B). When Mg^{2+} was used in poly rA•p(dT)$_{\overline{16}}$ reaction mixtures, the optimum concentration of Mg^{2+} was 1.5 mM, but the amount of enzyme activity was only 20% of the maximum with Mn^{2+} (Fig. 1B).

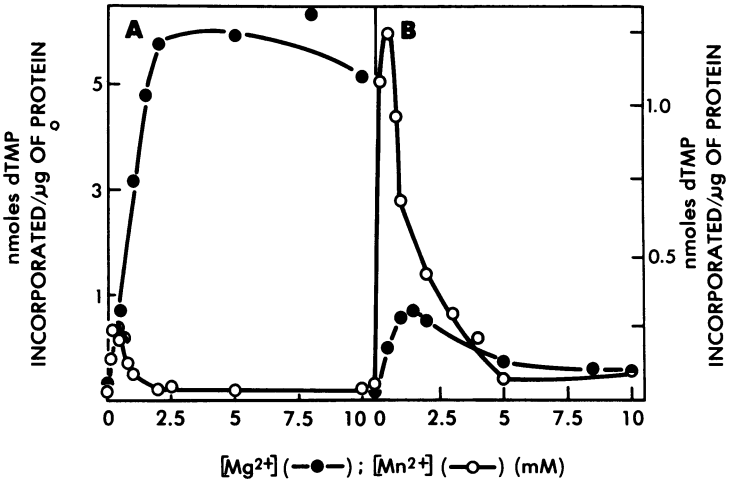

Fig. 1. Divalent cation requirements for DNA polymerase activities. Reactions were performed at 35°C for 15 min as described under Methods, except that the indicated concentrations of either $MgCl_2$ (—●—) or $MnCl_2$ (—○—) were used. In A, DNA polymerase activity with poly dA•p(dT)$_{\overline{16}}$ was determined using 2.5 units of Step 6 enzyme for each reaction. In B, DNA polymerase activity with poly rA•p(dT)$_{\overline{16}}$ was determined using 34 units of enzyme for each reaction.

Effects of Monovalent Cations and Spermidine on Enzyme Activity

DNA polymerase activities with poly dA•p(dT)$_{\overline{16}}$ and with poly rA•p(dT)$_{\overline{16}}$ were affected differently by K^+ ion (Fig. 2A). The enzyme activity with poly dA•p(dT)$_{\overline{16}}$ was strongly inhibited by added K^+ at concentrations above 30 mM: activity was reduced to 50% by 60-70 mM K^+ and was completely inhibited by 120 mM K^+ (Fig. 2A). However, in poly rA•p(dT)$_{\overline{16}}$ -directed poly dT synthesis, added K^+ stimulated enzyme activity, with a maximum effect at 80 mM K^+. Enzyme activity increased approximately 3-fold between 20 and 80 mM K^+, decreased to 66% of maximum between 90 and 200 mM K^+, and was inhibited above 220 mM K^+ (Fig. 2A).

The addition of spermidine to the DNA polymerase reaction mixtures had effects similar to the addition of K^+ ion (Fig. 2B). With poly dA•p(dT)$_{\overline{16}}$, enzyme activity was unaffected by spermidine concentrations between 0.01 and 1.0 mM, but was inhibited by spermidine concentrations of 2.0 mM or more. Spermidine stimulated enzyme activity in poly rA•p(dT)$_{\overline{16}}$ reaction mixtures containing only 20 mM K^+. Enzyme activity with poly rA•p(dT)$_{\overline{16}}$ was stimulated 3-fold by 0.5 mM spermidine. At concentrations above 2.0 mM, enzyme activity was severely inhibited, as with poly dA•p(dT)$_{\overline{16}}$.
Although spermidine and K^+ stimulated enzyme activity with poly rA•p(dT)$_{\overline{16}}$ as much as 3-fold when they were added separately, no further stimulation occurred when they were added together at their optimum concentrations (10).

Dependence of Enzyme Activity on the Concentration of Primer-Template and on the Ratio of Primer to Template. The incorporation of [^{3}H]dTMP was dependent on the concentration of added primer-template and, in both reaction mixtures, enzyme activity was maximum at template nucleotide concentrations of about 100 μM. The Michaelis constants were similar in both reactions (21 μM dAMP for poly dA•p(dT)$_{\overline{16}}$ reactions and 27 μM rAMP for poly rA•p(dT)$_{\overline{16}}$ reactions). In those reaction mixtures the ratio of primer nucleotides to template nucleotides was held constant at 1:10. When this ratio was varied, the rate of [^{3}H]dTMP incorporation decreased linearly up to a ratio of 1:10 and then remained relatively constant up to a ratio of 1:1, with both poly dA and poly rA templates. No [^{3}H]dTMP was incorporated with either poly dA or poly rA in the absence of the primer, or with p(dT)$_{\overline{16}}$ in the absence of a template.

Rate and Extent of Poly dT Synthesis. The rate of poly dT synthesis on the poly dA template increased linearly with enzyme protein concentration, and the rate of poly dT synthesis on the poly rA template increased in a sigmoidal manner with enzyme protein concentration (Fig. 3A). The synthesis

of poly dT at 35°C was linear for 60 min and continued at a reduced rate (approximately 50%) for at least 5 hr (Fig. 3B, C). The enzyme reactions eventually terminated under the conditions used in Figure 3C, although the enzyme retained 20-30% of its original activity after 24 hr at 35°C. With poly dA•p(dT)$_{\overline{16}}$, the synthesis of poly dT was complete after 5 hr of incubation, and no degradation of poly dT occurred over the 24 hr incubation

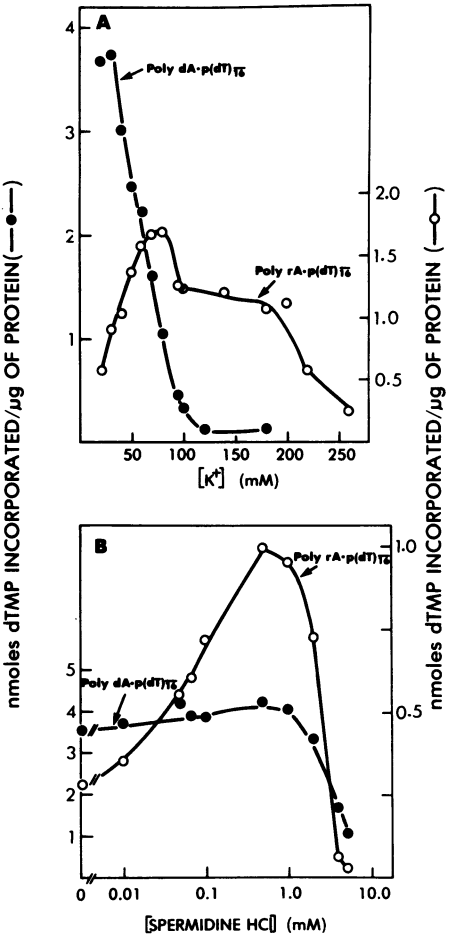

Fig. 2. Monovalent cation requirements and the effect of spermidine on enzyme activity. A. DNA polymerase reaction mixtures were adjusted to the indicated concentration of K^+ ion by adding KCl and DNA polymerase activity was assayed for 15 min at 35°C using 10 units of Step 6 DNA polymerase for poly dA•p(dT)$_{\overline{16}}$ reactions (—●—), or 15 units of enzyme for poly rA•p(dT)$_{\overline{16}}$ reactions (—○—). B. Spermidine HCl (Sigma) was added to DNA polymerase reaction mixtures at the indicated concentrations. Reaction mixtures were incubated for 15 min at 35°C. For poly dA•p(dT)$_{\overline{16}}$ reactions (—●—), 1.25 units of Step 6 DNA polymerase were used and for poly rA•p(dT)$_{\overline{16}}$ reactions (—○—), 15 units of enzyme were used. In both mixtures, the K^+ ion concentration was 20 mM.

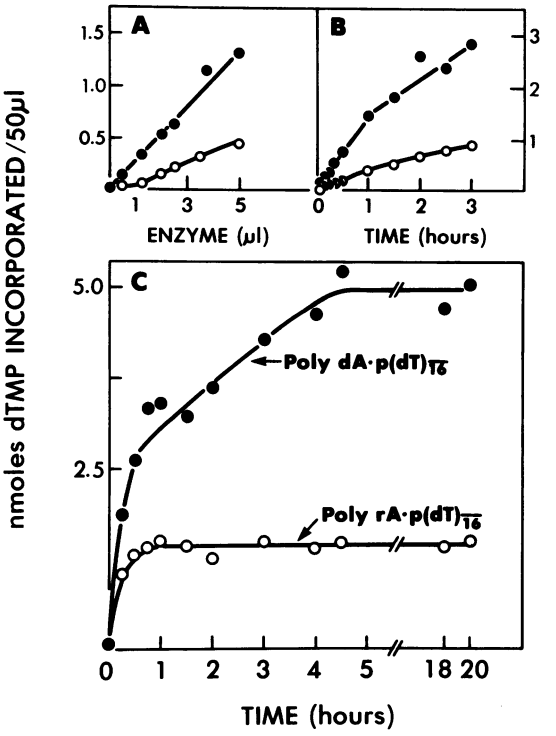

Fig. 3. Enzyme protein and time dependence of poly dT synthesis. A. DNA polymerase reaction mixtures were incubated for 15 min at 35°C with the indicated amounts of a Step 7 enzyme preparation, which had a specific activity of 52,000 units/mg of protein with poly dA·p(dT)$_{16}$. The incorporation of dTMP into poly dT in 50 µl standard reaction mixtures was determined with poly dA·p(dT)$_{16}$ (—●—) or poly rA·p(dT)$_{16}$ (—○—). B. Large, standard DNA polymerase reaction mixtures (0.6 ml total volumes) were incubated at 35°C and 50 µl portions were withdrawn at the indicated times after the beginning of the incubation and the incorporation of dTMP was determined. In poly dA·p(dT)$_{16}$ reactions (—●—), Step 7 DNA polymerase was added at 1.8 units/50 µl of reaction mixture and in poly rA·p(dT)$_{16}$ reactions (—○—) enzyme was added at 5 units/50 µl of reaction mixture. C. Large, standard DNA polymerase reaction mixtures (0.9 ml total volume) were incubated at 35°C and the amount of dTMP incorporated into poly dT in 50 µl portions was determined at the indicated times. Step 7 DNA polymerase was added at 5 units/50 µl to poly dA·p(dT)$_{16}$ reactions (—●—) and at 8 units/50 µl to poly rA·p(dT)$_{16}$ reactions (—○—).

period. With poly rA·p(dT)$_{16}$, the synthesis of poly dT was complete after 60 min of incubation at 35°C, and no loss of poly dT occurred during the 24 hr incubation at that temperature (Fig. 3C). At the completion of the poly dA·p(dT)$_{16}$ reaction, the maximum possible amount of poly dT had been synthesized; i.e., approximately 5 nmol of dTMP was incorporated into poly dT in each 50 µl reaction mixture. At the completion of the poly rA·p(dT)$_{16}$

reaction, the amount of poly dT synthesized was approximately one-third the amount of poly rA template.

Effect of Temperature on the Rate of Poly dT Synthesis. At 10°C, the initial rate of poly dT synthesis was identical for both primer-templates. The initial rate of poly dT synthesis increased 40-fold with poly dA•p(dT)$_{\overline{16}}$ and 12-fold with poly rA•p(dT)$_{\overline{16}}$ when the temperature of incubation was increased from 10°C to 35°C. With poly dA•p(dT)$_{\overline{16}}$, the rate of poly dT synthesis increased about 5- to 6-fold between 10°C and 25°C and 7-fold between 25°C and 35°C. However, with poly rA•p(dT)$_{\overline{16}}$, the reaction rate increased only 4-fold between 10°C and 25°C and only 3.3-fold between 25°C and 35°C. At 45°C the rate of poly dT synthesis was only slightly greater (1.3-fold) than it was at 35°C with poly dA•p(dT)$_{\overline{16}}$ and was lower (by about 10%) than it was at 35°C with poly rA•p(dT)$_{\overline{16}}$. The increase and decrease of reaction rates at 45°C may reflect the differential stability of the two enzyme activities to incubation at 45°C (see below). The activation energy for the enzyme reaction with poly dA•p(dT)$_{\overline{16}}$ was 31.7 kcal/mol, and the activation energy for the enzyme reaction with poly rA•p(dT)$_{\overline{16}}$ was 16.6 kcal/mol.

Inhibition and Inactivation of DNA Polymerase Activity. Inhibition of DNA polymerase activity with MalNEt was both time and concentration dependent and the activity of the enzyme with both primer-templates was inhibited similarly (Fig. 4A). When MalNEt was added at the beginning of the enzyme reactions, no effect was observed with MalNEt concentrations below 2.0 mM. Under these conditions, complete inhibition of enzyme activity occurred between 5 and 10 mM MalNEt (results not shown). When the DNA polymerase was pre-incubated with MalNEt for 30 or 60 min (at 0°C), partial inhibition of DNA polymerase activity was observed with concentrations of MalNEt as low as 0.1 and 0.5 mM (Fig. 4A). Longer pre-incubation periods (about 2 hr) were required to achieve complete inhibition of activity at these low concentrations of MalNEt. The results indicate that, with both primer-templates, 50-60% of the DNA polymerase activity was inhibited by very low concentrations of MalNEt and the remaining activity was more resistant to inhibition by MalNEt.

When the DNA polymerase was heated at 45°C, its activity with poly dA•p(dT)$_{\overline{16}}$ was more resistant to inactivation than was its activity with poly rA•p(dT)$_{\overline{16}}$ (Fig. 4B). DNA polymerase activity with poly dA•p(dT)$_{\overline{16}}$ was reduced to 50% after 15-17 min at 45°C and activity with poly rA•p(dT)$_{\overline{16}}$ was reduced to 50% after only 4-5 min at 45°C. In both cases, a reproducible

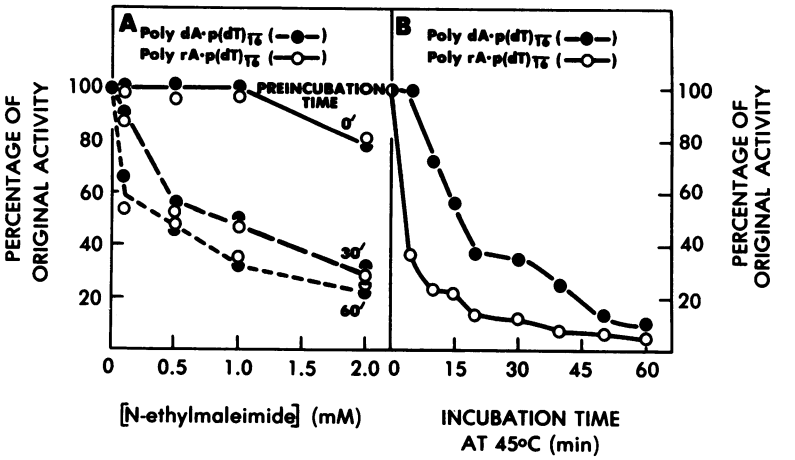

Fig. 4. Inhibition and inactivation of DNA polymerase activity. A. Step 6 DNA polymerase (30 units, 2 µg of protein) was incubated for 0, 30, or 60 min at 0°C with increasing concentrations of N-ethylmaleimide (MalNEt) before DNA polymerase activity was determined for 15 min at 35°C in reaction mixtures containing poly dA·p(dT)$_{16}$ (—●—) or poly rA·p(dT)$_{16}$ (—○—). B. Step 6 DNA polymerase was incubated at 45°C in a solution containing 50 mM Tris HCl (pH 8.5), 0.5 mM dithiothreitol, 0.1 mM EDTA, 20% (v/v) glycerol and 1 mg/ml bovine serum albumin. At the indicated times, 25 µl portions were removed to test tubes which were kept at 0°C until the 45°C incubation was completed (60 min). DNA polymerase substrates were added to the enzymes and the amount of DNA polymerase activity was determined for 15 min at 35°C. In poly dA·p(dT)$_{16}$ reactions (—●—), 2.3 units of enzyme were used for each determination and in poly rA·p(dT)$_{16}$ reactions (—○—), 9 units of enzyme were used for each determination.

plateau in the inactivation of enzyme activity separated the initial, rapid inactivation from the later, slow inactivation. The inactivation curves were logarithmic before and after the plateau regions. The rates of inactivation before and after the plateau differed 4-fold for poly dA·p(dT)$_{16}$ activity and 10-fold for poly rA·p(dT)$_{16}$ activity. These data suggest that heat inactivation of the enzyme activity occurred in several steps and that the enzyme may pass through a heat stable state during inactivation.

DISCUSSION

We have shown in this report that the activities of the Drosophila DNA polymerase with poly dA·p(dT)$_{16}$ and poly rA·p(dT)$_{16}$ were identical in their primer-template concentration dependence, their primer:template ratio dependence, and their MalNEt sensitivities. We have previously shown that the pH optimum was pH 8.5 for both enzyme reactions (10). This optimum was identical to that reported for a 5.5S Drosophila DNA polymerase (8) that was produced proteolytically from the 7.3S Drosophila DNA polymerase (9),

but differed from those of DNA polymerases-β and -γ. DNA polymerase-β is most active at pH 8.5 to 9.0 with activated DNA and with poly rA•oligo dT (3,14), and it is most active at pH 7.6 with poly dA•oligo dT (15). DNA polymerase-γ is maximally active between pH 7.0 and 7.6 with poly rA•p(dT)$_{\overline{16}}$ and between 7.9 and 8.3 with poly dA•p(dT)$_{\overline{16}}$ (12). In these two examples the two template activities were attributed to a single enzyme. The similarities of the two enzyme activities of the Drosophila DNA polymerase and the co-purification of the two activities (10) indicate that the same active site is involved in replication of both the deoxy- and the ribohomopolymer templates, although different mechanisms may be involved (see below).

The activities of the Drosophila DNA polymerase with poly dA•p(dT)$_{\overline{16}}$ and poly rA•p(dT)$_{\overline{16}}$ were differentiated by the extents of synthesis, the dependence of rates on enzyme concentration, the activation energies, heat inactivation kinetics, and the effects of divalent cations, K^+ ion, and spermidine. Some of these differences may have been due in part to the properties of the templates. For example, the different extents of synthesis with the two templates could have resulted from the lower base-pairing stability of the poly dA•poly dT complex, which allowed "slippage" of the product and caused more product to be synthesized. However, the effects of the divalent cations (Mg^{2+} and Mn^{2+}) on poly dT synthesis (Fig. 1 A,B) may result from the effects of these ions on the enzyme and on the enzyme-substrate interactions. Mn^{2+} ion has been shown to affect the fidelity of DNA polymerases, causing misincorporation of deoxynucleotides by vertebrate DNA polymerases (16,17) and increasing the incorporation of ribonucleotides by Escherichia coli DNA polymerase (18), indicating that divalent cations affect enzyme-template-deoxynucleotide interactions. The divalent cation preferences reported here are similar to those observed for the Drosophila DNA polymerase purified by Karkas et al. (8), for a Tetrahymena DNA polymerase (4), and for DNA polymerase-γ (12). However, DNA polymerase-β preferred Mn^{2+} ion with both of the primer-templates used in our studies (1,13,14,16).

It is probable that K^+ ion affected both primer-templates similarly, but differentially affected the activities of the Drosophila DNA polymerase (Fig. 2A). The effect of K^+ on the Drosophila DNA polymerase activities was similar to that reported for the mouse myeloma DNA polymerase-γ (12), but was different from that reported for the KB cell DNA polymerase-β (13). It also differed from the effect of Na^+ on the activity of a human liver DNA polymerase-β with poly dA•oligo dT and poly rA•oligo dT (14). The

effects of K^+ on the activities of the Drosophila DNA polymerase may be related to its effect on the physical state of the enzyme. The Drosophila DNA polymerase is a heterogeneous mixture of aggregated (> 7.3S) and unaggregated (7.3S) enzyme molecules at low K^+ concentrations (50 mM) and is unaggregated (7.3S) at high K^+ concentrations (100 mM or higher) (C.L.B., unpublished observations, 1975). The inhibition of activity with poly dA•p(dT)$_{\overline{16}}$ and the stimulation of activity with poly rA•p(dT)$_{\overline{16}}$ by K^+ (Fig. 2A) suggest that replication of poly dA may be facilitated by an association of DNA polymerase molecules and that poly rA replication may be carried out by unassociated enzyme molecules. This hypothesis may explain the different heat stabilities of the enzyme activities and the different activation energies of the reactions. In low ionic strength buffers, poly dA replication activity was more stable to incubation at 45°C than poly rA replication activity. This result suggests an increased thermal stability of associated enzymes relative to unassociated enzymes (Fig. 4B). The higher activation energy of synthesis of poly dT on poly dA, compared to poly rA, may be due to the requirement for more polymerase molecules in the former reaction.

The behavior of the 7.3S Drosophila DNA polymerase described in this report distinguishes the enzyme from vertebrate DNA polymerases-α and -β and indicates its similarity to vertebrate DNA polymerase-γ and to the Tetrahymena DNA polymerase (4). A comparison of the activities of the different forms of the Drosophila DNA polymerase (9,10) with these two primer-templates should allow us to define the structure-function relationships of the different enzyme forms; e.g., the 5.5S trypsin-cleavage product of the 7.3S enzyme, which retains full polymerizing activity although it has lost a 50,000 dalton peptide(s) of undefined function (9).

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