Volume 12 Number 14 1984 Nucleic Acids Research

The effect of the 3',5' thiophosphoryl linkage on the exonuclease activities of T4 polymerase and the Klenow fragment

Amar P.Gupta, Patricia A.Benkovic and Stephen J.Benkovic

Department of Chemistry, Pennsylvania State University, 152 Davey Laboratory, University Park, PA 16802, USA

Received 16 April 1984; Revised and Accepted 17 June 1984

ABSTRACT

The 3'→5' exonuclease activities of T4 DNA polymerase and the Klenow fragment of Polymerase I towards the phosphoryl and thiophosphoryl 3',5' linkage were examined under comparable conditions of idling-turnover, duplex hydrolysis and turnover during polymerization. With the T4 enzyme there is a negligible effect of thiosubstitution on these activities; with the Klenow fragment there is a greater than one hundred-fold reduction in rate with the thiolinkage for the exonuclease but not polymerization activities. This inability to hydrolyze rapidly the thiophosphoryl linkage extends to the hydrolytic activity of Exonuclease III. The quantitation of the exonuclease activities of these three proteins under various conditions should aid in the successful employment of thiophosphoryl nucleoside triphosphates for their incorporation into DNA.

INTRODUCTION

Prokaryotic DNA polymerases possess inherent 3'→5' exonuclease activities. As a consequence these enzymes can act as bona fide exonucleases, capable of hydrolysing both single stranded and duplex DNA (1,2). The 3'→5' exonuclease activity seems to originate from the same domain on the enzyme as the polymerase function so the two activities are closely related. The close association of the polymerase and exonuclease activities is evident in their simultaneous expression either during polymerization, resulting in a certain fraction of the deoxynucleoside triphosphate pool being converted into the corresponding monophosphate or during idling-turnover where successive cycles of incorporation and excision result in conversion of the entire triphosphate pool into the monophosphate (3,4). A convincing argument for an inverse relationship between the two activities comes from the work of Das and Fujimura who showed that T5 DNA polymerase on a given enzyme-template-primer complex could switch from an exonuclease to a polymerase function (5). One popular rationale for the existence of exonuclease activities in DNA polymerases is that this activity is responsible for the high fidelity of replication by deleting mismatches when they

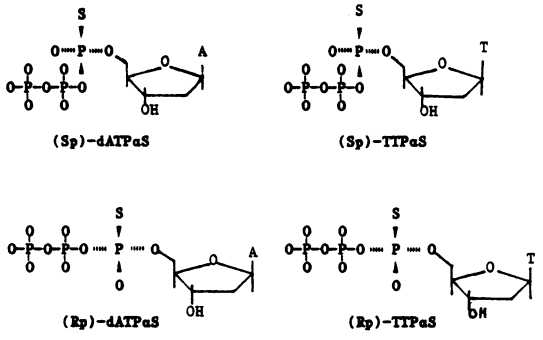

Figure 1. Structures for dATPαS and TTPαS stereoisomers.

occur. Considerable evidence to support this hypothesis exists (6-12).

In the work described here, the exonuclease activities of two enzymes, T4 DNA polymerase and the Klenow Fragment of Pol I are systematically examined. Using poly[d(A-T)], deoxynucleoside triphosphates and their α-thioanalogs (Figure 1) at saturating concentrations, and a constant catalytic concentration of enzyme, initial rates for different modes of exonuclease action and polymerization were determined. Under these conditions, several different rate comparisons can be made directly. A comparison of exonuclease rates for the oxy- and thiophosphoryl substrates during idling-turnover, hydrolysis, and turnover during polymerization provides information for a given enzyme about the effect of deoxynucleoside triphosphate cosubstrates on the exonuclease activity and the rate limiting steps in various processes. The exonuclease activities of T4 polymerase and DNA polymerase I then are contrasted revealing a fundamental mechanistic difference between the two enzymes.

MATERIALS AND METHODS

Enzymes

T4 DNA Polymerase (T4 am N82-infected *E. coli* B) was purchased from P-L Biochemicals. The enzyme had a specific activity of 20,000 units/mg. The enzyme was judged to be sufficiently free of contaminating endonucleases to be used in these experiments by the following criterion: Upon incubation of 1.0 μg of φX174 form I DNA with 2 units of enzyme in a 10 μl reaction mixture containing 50 mM Tris.HCl (pH 7.6), 10 mM Mg^{2+}, and 1 mM β-mercaptoethanol at 37°C for 1 h, no detectable loss of supercoiled DNA was observed as determined by agarose gel electrophoresis. In order to insure that the hydrolytic reactions described here were not due to some

contaminating exonuclease activity, an idling-turnover experiment was repeated using highly purified T4 DNA polymerase obtained courtesy of Dr. Nancy Nossal. The ratio of initial rates of formation of dAMP and dAMPS was found to be identical to the ratio obtained by the commercial enzyme.

DNA Polymerase I (E. coli) 'Klenow fragment' was purchased from P-L Biochemicals. The enzyme had a specific activity of 7500 units/mg. The enzyme was assayed for the 5'→3' exonuclease activity using the poly[d(A-T)]-primed hypo-hyperchromic assay of Setlow et. al. (13). Less than a 5% hyperchromic increase was observed during a period of time corresponding to twice the time of the hypochromic phase of the reaction.

Exonuclease III (E. coli) was purchased from P-L Biochemicals. The enzyme had a specific activity of 178,000 units/mg. Incubation of 400 u/ml of exonuclease III in a 10 µl reaction containing 150 µg/ml ϕX174 Form I DNA, 0.05 M Tris.HCl (pH 8), 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol at 37 °C for 30 minutes showed no change in the amount of supercoiled Form I DNA remaining relative to a no-enzyme control thus indicating the lack of endonuclease activity.

DNase I, adenylate kinase and pyruvate kinase were purchased from Sigma Chemicals.

Nucleotides and Nucleic Acids

^{3}H-dATP, sp.act. 70 Ci/mmole, was purchased from ICN. The commercial radionucleotide contained ~17.5% of its radioactivity in dAMP, dADP and other unidentified impurities as determined by a TLC assay on PEI-cellulose (see below). The compound was purified by anion exchange chromatography on a DEAE-Sephadex A-25 column (0.5 x 12.5 cm) with a linear gradient of 200 ml TEAB buffer from 0.2 to 0.5 M. ^{3}H-dATP was eluted at a buffer concentration of 0.34 M. Non-radioactive deoxynucleoside triphosphates were from Sigma. ^{35}S-(Sp)-dATPαS was purchased from New England Nuclear and had an initial specific activity of 1202 Ci/mmol. The compound was ascertained to be of greater than 99% radiochemical purity by TLC assay (see below) and used without further purification. Non-radioactive Sp-dATPαS was synthesized by the method described previously (4). The synthesis of (Sp)-TTPαS is described under Methods. Poly[d(A-T)] was purchased from P-L Biochemicals. Poly[d(A-T)][^{3}H-dA]$_{1.0}$ and poly[d(A-T)][^{35}S-dA]$_{1.0}$ were prepared by 3'-end labeling of poly[d(A-T)] as described in Methods. Liquiscint was purchased from National Diagnostics.

METHODS

Synthesis of (Sp+Rp)TTPαS

Thymidine (242 mg, 1 mmole) was dried overnight in a drying-pistol over P_2O_5 at 57°C. Further drying was effected by several evaporations with anhydrous pyridine. The resulting amorphous white powder was dissolved in 2.5 ml triethyl phosphate which had been freshly distilled over calcium hydride. Slight warming was required to accomplish complete dissolution of thymidine. The clear solution was cooled down to 0°C in an ice-bath and $PSCl_3$ (120 µl, 1.2 mmoles) was added. The ice-bath was removed and the reaction mixture stirred at room temperature. An additional 100 µl (1 mmole) of $PSCl_3$ was added after 18 hours and the mixture stirred for an additional 12 hours. At the end of this period, the reaction flask was connected to a vacuum pump and the excess $PSCl_3$ pumped off. Triethylammonium bicarbonate (1 M, 10 ml) was added and the mixture stirred for one hour. The excess TEAB was removed by coevaporation with ethanol; the resulting solution was diluted with 20 ml H_2O and applied to a DEAE cellulose column (2 x 40 cm, HCO_3^- form) and eluted with a 1000 ml linear gradient of 0-0.3 M TEAB. There were 100 fractions collected (10 mL each) with TMPS eluting at 0.12 M Buffer. This procedure was repeated and TMPS free of inorganic thiophosphate was found in fractions 38-45. These were pooled and evaporated; the TEAB was removed by coevaporations with ethanol. A total of 265 µmoles TMPS (27% yield) was obtained. The synthesis of (Sp+Rp)TTPαS was accomplished by employing the method described for the synthesis of ATPαS (14). The diastereomers were resolved by column chromatography as described in ref. 15 with the exception that ammonium bicarbonate was employed as the eluant.

DNAase I Activation of Poly[d(A-T)]

The procedure described by Modrich and Lehman was used (16) to obtain poly[d(A-T)] of average effective chainlength ca. 1000.

Enzyme Assays

T4 Polymerase The reaction mixture contained per ml, 66.7 µmoles of Tris.HCl (pH 8.8), 16.7 µmoles of dithiothreitol, 100 µg of BSA, 6.7 nmoles of EDTA, 6.7 µmoles of $MgCl_2$, 0.25 µmoles of poly[d(A-T)] and 100 nmoles each of either ^{3}H-dATP or ^{35}S-dATPαS. Where polymerization was being measured, 100 nmoles of TTP or TTPαS was included as indicated. For the 3'→5' exonuclease assay, the same conditions were employed except that poly[d(A-T)] was replaced by 0.25 µmoles of poly[d(A-T)][^{3}H-dA]$_{1.0}$ or poly[d(A-T)][^{35}S-dA]$_{1.0}$ and deoxynucleoside triphosphates or analogs were

omitted. The concentration of T4 polymerase was 8.9 nM unless otherwise indicated. Incubations were at 37°C.

Klenow Fragment The reaction mixture contained per ml, 60 µmoles of potassium phosphate (pH 7.4), 1 µmole of β-mercaptoethanol and 6.7 µmoles of $MgCl_2$. The other conditions were the same as for the T4 polymerase except that the enzyme concentration was 88 nM.

Exonuclease III The reaction mixture contained per ml, 66.7 µmoles of Tris.HCl (pH 8), 6.7 µmoles of $MgCl_2$, 10 µmoles of β-mercaptoethanol and 0.25 µmoles of poly[d(A-T)][^{3}H-dA]$_{1.0}$ or poly[d(A-T)][^{35}S-dA]$_{1.0}$. The enzyme concentration was 10 units/ml and the incubation was at 37°C.

TLC Assay for Monophosphate Generation

PEI cellulose plates with fluorescent indicator were employed to follow the formation of deoxynucleoside monophosphates (or phosphorothioates) during idling or polymerization. A 20 cm x 20 cm TLC plate was divided into 20 lanes, each 1 cm wide. The origin (1.5 cm above bottom edge) was pre-spotted with 3 µl of a quenching solution of .1 M EDTA and 3 µl of a UV-visualization marker solution (4 mM dAMP, 2 mM each of dADP and dATP for the ^{3}H-dATP reactions and 2 mM each of dAMPS and dATPαS for the ^{35}S-dATPαS reactions.) For each time point, a 5 µl aliquot of the reaction mixture was spotted at the origin. The TLC plate was then developed in either 1 M KCl or 1.2 M LiCl, allowed to dry and after UV visualization the monophosphate and the triphosphate spots were cut out. The TLC pieces were placed directly into scintillation vials. When counting ^{35}S, 10 ml Liquiscint was added and the radioactivity counted in a Beckman LS8100 Liquid Scintillation Counter. When counting ^{3}H, the radioactivity was first eluted off by soaking the pieces of TLC plate in 1 ml of 1N HCl for 10 minutes, 10 ml of Liquiscint was then added and the radioactivity counted as above.

Polymerization Assays

Incorporation of ^{35}S-dAMPS into polymer was followed by measuring the retention of radioactivity on DE81 paper circles (6). For each time point, a 5 µl aliquot was placed in the center of a DE81 filter paper circle (2.4 cm dia, Whatman) which had been pre-spotted with 10 µl of 0.1 M EDTA as a quench. After drying, these papers were then washed 3 times by gentle agitation for 20 mins in 0.3 M ammonium formate (pH 8.0). The papers were then washed twice in 95% ethanol and once in diethyl ether. The papers were allowed to air-dry and then were counted in 5 ml of a standard toluene scintillation fluid. This procedure removes nucleoside

triphosphates from the papers while leaving polymer bound. In some assays, incorporation of ^{3}H-dAMP into polymer was also followed by this method and the results were found to be identical with those obtained by the acid-precipitation method (17).

Hydrolysis Assays

Exonucleolytic hydrolysis in the absence of deoxynucleoside triphosphates was also measured by a DE81 paper method (18). For each time point, a 10 µl aliquot of the incubation mixture was placed on a DE81 paper prespotted with 10 µl of 0.1 M EDTA. The filter was air dried for 30 minutes and then placed in a scintillation vial. It was then washed by gentle agitation for 15 min with one ml of ammonium formate (0.3 M, pH 7.5) followed by two washings with 0.5 ml of the same solution. The combined washings were mixed with 15 ml Liquiscint scintillation cocktail and counted as above.

3'-Terminal-labelling of Poly[d(A-T)]

Poly[d(A-T)] was 3'-end labelled with ^{3}H-dAMP or ^{35}S-dAMPS to prepare substrates for comparing 3'→5' exonuclease activities under hydrolytic conditions. The procedure also served as a means for chain length determination of poly[d(A-T)]. The reaction mixture contained in a total volume of 1 ml: 66.6 µmoles of Tris.HCl (pH 8.8), 16.7 µmoles of dithiothreitol, 100 µg of BSA, 6.7 nmoles of EDTA, 6.7 µmoles of $MgCl_2$, 0.25 µmoles of poly[d(A-T)] and either 25 nmoles of ^{3}H-dATP (sp.act. 772 cpm/pmole) or 25 nmoles of ^{35}S-dATPαS (sp.act. 795 cpm/pmole). The reaction was started by the addition of 4.4 pmoles of T4 polymerase and the mixture was incubated at 21°C for 30 minutes. The reaction was stopped by adding 100 µl of 0.1 M EDTA and the enzyme was inactivated by heating at 75°C for 10 minutes followed by rapid cooling in an ice bath. The 3'-end labelled poly[d(A-T)] was isolated by dialyzing the resulting solution at 4°C against 1 L of 0.15 M NaCl + 0.015 M sodium citrate (pH 7) for 24 hours, followed by three changes of 2 L of 0.015 M NaCl + 0.0015 M sodium citrate (pH 7) for 12 hours each. A total of 1.33 A_{260} absorbance units of polymer in 1.1 ml was recovered from the dATPαS reaction. A 10λ aliquot was mixed with 10 ml Liquiscint and counted as above. Based on an ε_{260} of 6.8 for poly[d(A-T)] and a specific activity for the starting ^{35}S-dATPαS of 810 cpm/pmole, the labelled poly-[d(A-T)] was found to contain 1.12 pmole of ^{35}S-dAMPS per nmole of total nucleotide in poly[d(A-T)]. Presuming each 3'-end is quantitatively labelled an average effective chain length of 890 for poly[d(A-T)] is calculated. From the ^{3}H-dATP reaction, a total of 1.39 A_{260} absorb-

ance units of polymer in 1.2 ml total volume was obtained and a similar analysis yielded an average chain length of 945 for the poly[d(A-T)][^{3}H-dA] product.

RESULTS

Idling-turnover with T4 DNA Polymerase

Both dATP and (Sp)-dATPαS were rapidly converted into dAMP and dAMPS respectively when incubated at a concentration of 0.1 mM in the presence of 0.25 mM poly[d(A-T)] and 8.9 nM T4 DNA polymerase (Figure 2). This conversion is believed to take place via repeated incorporation-excision cycles of the deoxynucleotide or the thioanalog into poly[d(A-T)] (4,19-21). Appropriate controls were carried out to establish that this turnover is indeed template-primer dependent and that incorporation is a prerequisite; namely, (Rp)-dATPαS which is inactive in the polymerization reaction (22) is also inactive in idling-turnover. The lack of turnover in the absence of poly[d(A-T)] ruled out any dATPase type activities. Further evidence was obtained to support the incorporation-excision model for turnover by following incorporation of ^{35}S-dAMPS into poly[d(A-T)] during the course of turnover. Quenching the reaction at any time during the linear phase of

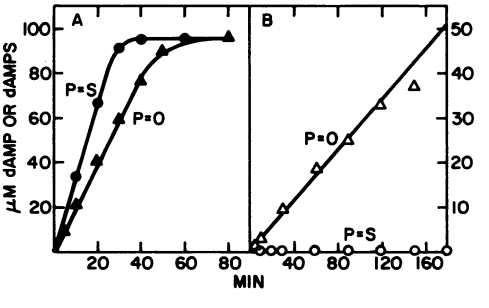

Figure 2. (A) Idling-turnover of dATP (▲) and dATPαS (●) with T4 DNA polymerase. A 100 µl reaction mixture containing 66.7 mM Tris.HCl (pH 8.8), 16.7 mM dithiothreitol, 100 µg/ml BSA, 6.7 µM EDTA, 6.7 mM $MgCl_2$, 0.25 mM poly[d(A-T)], 8.9 nM T4 DNA polymerase and 100 µM dATP or dATPαS was incubated at 37°C. Five microliter aliquots were withdrawn at various time intervals and analyzed by TLC assay as described under methods. (B) Idling-turnover of dATP (△) and dATPαS (○) with Klenow Fragment. A 100 µl reaction mixture containing 60 mM potassium phosphate (pH 7.4), 1 mM β-mercaptoethanol, 6.7 mM $MgCl_2$, 0.25 mM poly[d(A-T)], 88 nM Klenow Fragment and 100 µM dATP or dATPαS was incubated at 37°C. The amounts of monophosphate or monothiophosphate generated at different times were determined as in A.

turnover and examining the poly[d(A-T)] for incorporation of ^{35}S-dAMPS revealed a constant amount of incorporation that closely approximated the concentration of poly[d(A-T)], assuming one nucleotide incorporated per chain. Similar results were obtained for the incorporation of ^{3}H-dAMP. As seen in Figure 2 the rate of idling-turnover of dATPαS was almost twice as fast as that for dATP. The fact that quenching the reaction always yields poly[d(A-T)] labelled on a 1:1 basis at the 3'-end with either ^{3}H-dAMP or ^{35}S-dAMPS clearly indicates that the incorporation part of the incorporation-excision cycle must be the faster of the two processes and excision must be rate limiting. This would then indicate that 3' 5' exonucleolytic hydrolysis under these conditions is actually faster for the thiosubstrate than it is for the normal oxysubstrate.

Idling-turnover with Klenow Fragment

In contrast to the T4 DNA polymerase, the Klenow fragment of Pol I shows great discrimination between dATP and (Sp)-dATPαS as substrates for poly[d(A-T)] dependent turnover. As exhibited in Figure 2, when 0.1 mM dATP was incubated in the presence of 0.25 mM poly[d(A-T)] and 88 nM enzyme, it was converted into dAMP at the rate of 0.33 μM/min. Under these same conditions, no dAMPS formation was detected when (Sp)-dATPαS was employed as a substrate. Based on the limits of detection, this reflects a rate difference of at least 120:1. It had been shown earlier (23) and we reconfirm that the rate of polymerization with Pol I employing poly[d(A-T)] is neglibibly affected when (Sp)-dATPαS is substituted for dATP. It follows that the large rate difference observed must be a result of a greatly reduced 3'→5' exonucleolytic hydrolysis by the enzyme of the thiosubstrate.

Hydrolysis of poly[d(A-T)][^{3}H-dA]$_{1,0}$ and poly[d(A-T)]-[^{35}S-dA]$_{1,0}$ with T4 DNA Polymerase

For a direct comparison of exonucleolytic hydrolysis, DNA substrates are required that are identical in all respects except for the nature of the phosphodiester linkage whose hydrolysis is being measured. Such substrates are provided by the idling reaction which yields poly[d(A-T)] polymers differing only in the 3'-terminal label which may be ^{3}H-dA or ^{35}S-dA depending on whether ^{3}H-dATP or ^{35}S-(Sp)-dATPαS was employed as substrate. A direct test for the 3'→5' exonuclease activity of T4 polymerase toward the thiophosphoryl internucleotide linkage in the absence of deoxynucleoside triphosphates was made by comparing the rates of ^{35}S-dAMPS and ^{3}H-dAMP release from the 3'-end of poly [d(A-T)]. Since the only

Table I
Initial Rates with T4 DNA Polymerase

Mode	Substrate	[E] nM[a]	[S] μM[b]	Vo[c]
Idling	dATP	8.9	100[d]	225
Idling	(Sp)-dATPαS	8.9	100[d]	382
Hydrolysis	poly[d(A-T)][^{3}H-dA]$_{1.0}$	8.9	250	10.7
Hydrolysis	poly[d(A-T)][^{35}S-dA]$_{1.0}$	8.9	250	11.2
Incorporation	dATP + TTP	8.9	100[d]	438
Incorporation	dATPαS + TTP	8.9	100[d]	98
Turnover	dATP + TTP	8.9	100[d]	359
Turnover	dATPαS + TTP	8.9	100[d]	197

[a] Enzyme concentration, based on a molecular weight of 112,000. [b] Substrate concentration, for each nucleotide when more than one nucleotide is employed, or total nucleotide concentration for end-labeled poly[d(A-T)]. [c] Initial velocity expressed in moles of nucleotide/mole of enzyme/min. [d] Poly[d(A-T)] concentration was 250 μM.

difference between the two substrates is the nature of the 3'-end label, nonproductive binding of the enzyme cancels.

Figure 3 shows the rates of release of ^{3}H-dAMP and ^{35}S-dAMPS from the 3'-end of the corresponding polymers at substrate and enzyme concentrations identical to those in the idling and polymerization experiments so that these rates may be directly compared. From initial rates, the exonucleolytic hydrolysis of the poly[d(A-T)][^{3}H-dA]$_{1.0}$ is ca. twenty times slower than the rate of idling-turnover of dATP. Similarly, the rate of exonucleolytic hydrolysis of poly[d(A-T)][^{35}S-dA]$_{1.0}$ is ca. thirty-five fold slower than the rate of idling-turnover of dATPαS (Table I). Figure 3 also shows that the initial rates of hydrolysis of the thiophosphoryl and phosphoryl substrates are identical within experimental error.

The slower rate of hydrolysis as contrasted with the idling-turnover rate for either thiophosphoryl or phosphoryl substrate does not stem from a processive exonuclease action by T4 polymerase. Since only the terminal nucleotide is labelled, any degree of processivity would result in an underestimation of the exonuclease rate. However, considerable evidence

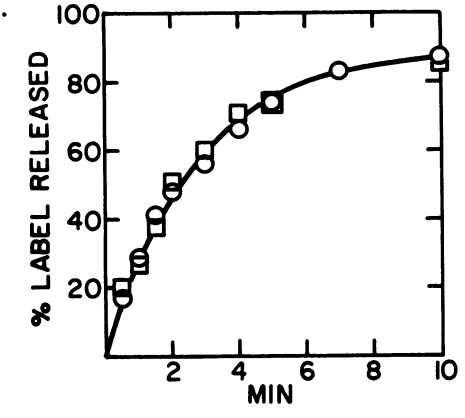

Figure 3. Hydrolysis of the terminal residue from poly[d(A-T)]-[^{3}H-dA]$_{1.0}$ and poly[d(A-T)][^{35}S-dA] with T4 DNA polymerase. A 100 µl reaction mixture containing 66.7 mM Tris.HCl (pH 8.8), 16.7 mM dithiothreitol, 100 µg/ml BSA, 6.7 µM EDTA, 6.7 mM $MgCl_2$, 8.9 nM T4 DNA polymerase and either 0.25 mM poly[d(A-T)][^{3}H-dA]$_{1.0}$ (O) or poly[d(A-T)][^{35}S-dA] (□) was incubated at 37°C. Ten microliter aliquots were withdrawn at various times and the amount of label released measured as described in Methods.

exists that exonucleolytic hydrolysis by T4 polymerase is indeed nonprocessive (24). Thus, the following rationales are offered: 1) Idling-turnover may not involve either translocation nor dissociation of enzyme whereas simple non-processive hydrolysis requires dissociation of the enzyme after each turnover. If dissociation is a slow step, an overall slower rate for 3'→5' hydrolysis relative to idling-turnover is possible. The identical rate of hydrolysis for oxy- and thiosubstrates is a very good indication that the chemical step is not rate limiting (25). 2) In contrasting hydrolysis with idling-turnover, a notable difference between the two processes is that in the latter, the exonuclease activity is being expressed in the presence of a triphosphate. It is plausible but less likely that interaction of the deoxynucleoside triphosphate with the enzyme enhances rather than inhibits the exonuclease activity.

Hydrolysis of poly[d(A-T)][^{3}H-dA]$_{1.0}$ and poly[d(A-T)]-[^{35}S-dA]$_{1.0}$ with Klenow Fragment

Table II records the rates of release of label from poly[d(A-T)][^{3}H-dA]$_{1.0}$ and poly[d(A-T)][^{35}S-dA]$_{1.0}$ with the Klenow fragment. The polymer concentrations were again 0.25 mM and the enzyme concentration was 88 nM. Comparing the rate of hydrolysis of poly[d(A-T)][^{3}H-dA]$_{1.0}$ with the rate of idling-turnover of dATP in the presence of an equivalent concentration of poly[d(A-T)] reveals that simple exonucleolytic hydrolysis is five times slower. The phenomenon of the idling process being faster than the rate for exonuclease activity for Pol I had been observed earlier (26). In

contrast to T4 polymerase, the rate of 3'→5' hydrolysis of the thiophosphoryl substrate was found to be 117 times slower than with the phosphoryl substrate. Thus the effect of thiosubstitution shows up in both idling-turnover and simple exonucleolytic hydrolysis with this enzyme and is expressed either in the presence or absence of deoxynucleoside triphosphates. In as much as the exonuclease activity plays an editing role, it should be possible to increase the mutational frequency observed with Pol I by employing thiosubstrates as has been recently demonstrated (27).

Hydrolysis of poly[d(A-T)][^{3}H-dA] and poly[d(A-T)][^{35}S-dA] with Exonuclease III.

No hydrolysis of the thiophosphoryl substrate was observed under our conditions. Based on our limits of detection the thiophosphoryl substrate must be hydrolysed at least 240 times slower. This observation confirms the results of Putney et.al (28), who first suggested capping 3'-ends of duplex DNA with thiosubstrates in order to protect them from hydrolysis by Exonuclease III.

Turnover During Polymerization with T4 Polymerase

It has been argued (3) that monophosphate generation during polymerization is the most relevant measure of the proofreading capacity of the polymerase since it measures hydrolysis in the presence of deoxynucleoside triphosphate substrates. Direct correlations between the amounts of monophosphate generated during polymerization and the contribution of proofreading activity to the overall fidelity of replication have been made (7-9,19,26,29).

Polymerization and turnover of either (Sp)-dATPαS or dATP at a conc. of 0.1 mM in the presence of 0.1 mM TTP, poly[d(A-T)] (0.25 mM) and 8.9 nM T4 polymerase were followed with time. The results are tabulated in Table I. The initial rate (data not shown) of incorporation of dAMPS is 4.5 times slower than that for dAMP incorporation. This reveals a larger effect of sulfur substitution on polymerization than observed with the Klenow fragment where the rate of dAMPS incorporation is only 1.4 times slower than dAMP incorporation (Table II). Even though the rate of turnover during polymerization with dATP is ca. 1.8 times as fast as that with dATPαS, the ratio of incorporation to turnover with (Sp)-dATPαS is only 0.5, in a clear contrast to dATP where the ratio is 1.2. Consequently proofreading should be enhanced and not diminished when using (Sp)-dATPαS. The >500 fold decrease in accuracy for T4 DNA polymerase in the φX174 fidelity assay (27) is inexplicable within this framework.

Table II
Initial Rates with Klenow Fragment

Mode	Substrate	[E] nM	[S] μM	Vo[a]
Idling	dATP	88	100[b]	3.4
Idling	dATPαS	88	100[b]	<0.03
Hydrolysis	poly[d(A-T)][^{3}H-dA]$_{1.0}$	88	250	0.73
Hydrolysis	poly[d(A-T)][^{35}S-dA]$_{1.0}$	88	250	6.6×10^{-3}
Incorporation	dATP + TTP	88	100[b]	124
Incorporation	dATPαS + TTP	88	100[b]	86

[a] Expressed in moles of nucleotide/mole of enzyme/min. [b] Poly[d(A-T)] concentration was 250 μM.

Replication of poly[d(A-T)] with T4 DNA Polymerase Using (Sp)-dATPαS and (Sp)dTTPαS.

Contrary to expectation, the replacement of both dATP and TTP with thioanalogs during replication of poly[d(A-T)] gives a small burst of incorporation corresponding to approximately two residues of dAMPS per chain followed by a complete lack of further polymerization (Fig.4). The turnover of dATPαS during polymerization shows two distinct phases. An initial fast rate corresponding to the incorporation phase is followed by a slower rate during the phase of no net polymerization.

Several possible explanations for this behavior were investigated. The possibility that the enzyme was irreversibly inactivated was ruled out

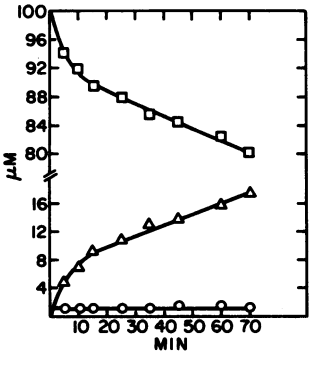

Figure 4. Replication of poly[d(A-T)] with ^{35}S-dATPαS and TTPαS with T4 polymerase. Both substrates were present at a concentration of 100 μM. Other conditions were the same as in Fig.2. Incorporation was measured by counting the radioactivity at the origin in the TLC assay. O-Incorporation, Δ-turnover, □-dATPαS utilization.

because the template-primer dependent turnover of dATPαS continues at a steady rate until all the dATPαS is exhausted. Addition of Pol I or Klenow fragment after net synthesis has stopped, results in a return to a normal rate of synthesis, so that the lack of continued synthesis by the T4 enzyme reflects an anomaly in the poly[d(A-T)]·T4 enzyme complex. This interpretation is supported by the observation that the polymerase can replicate the polydT· oligodA system in a normal fashion. Apparently the structure of poly[d(A-T)] [P_Sd(A-T)] impairs continued elongation by the T4 enzyme which is known to be highly sensitive to the secondary structure of its template-primer.

DISCUSSION

A Comparison of Pol I and T4 Polymerase

As noted above and as seen from the data summarized in Table III, T4 polymerase shows no or a small effect of thiosubstitution on its activities. It would appear therefore, that neither in the polymerization, nor in the exonuclease reaction, does the reaction sequence involve a rate determining nucleophilic attack on the phosphoryl center. Since the rate of the idling process which combines polymerization and excision reactions exceeds that for the simple non processive 3'→5' hydrolysis, it is possible that the latter is limited by dissociation/reassociation of the enzyme with the 3'-terminus of the substrate, whereas idling may represent multiple turnover events before enzyme dissociation. Furthermore, since the idling process generates 3'-ends stoichiometrically labelled, the polymerization reaction component--incorporation of a single NMP--must be at least

Table III
A Comparison of P-O/P-S Effects on the Rates of Various Excision Activities.

Enzyme	Relative Rates: Idling[a] (P-O)	Idling[a] (P-S)	3'-5' hydrolysis[b] (P-O)	3'-5' hydrolysis[b] (P-S)	Turnover[c] (P-O)	Turnover[c] (P-S)
T4 Polymerase	1.0	1.7	0.045	0.045	1.6	0.85
Klenow Pol I	1.0	<0.01	0.20	0.0017	-	-
Exonuclease III	-	-	1.0	<0.005	-	-

[a] dATP or dATPαS substrate; poly[d(A-T)] template. [b] Poly[d(A-T)][^{3}H-dA]$_{1.0}$ or Poly[d(A-T)][^{35}S-dA]$_{1.0}$ substrate. [c] Conversion of dATP to dAMP or dATPαS to dAMPS in the presence of the poly[d(A-T)] template and TTP cosubstrate.

ten-fold faster than excision. This means that the rate of polymerization associated with the idling process is considerably faster than the rate of polymerization associated with incorporation and suggests that the latter is likewise limited by either translocation or dissociation/reassociation of the enzyme. The same arguments probably are valid for Pol I since its lower idling rate may conceal its true rate of incorporation of a single NMP.

In the case of Pol I, there is no effect of thiosubstitution in the polymerization reaction but all the manifestations of the exonuclease function show the characteristic greater than hundred-fold reduction in rate with thiosubstrates (Table III). This inability to hydrolyze rapidly the thiophosphoryl linkage extends to the hydrolytic activity of Exonuclease III. Whether this represents a fundamental difference in mechanism between the T4 exonuclease and the others is under investigation. Nevertheless, the successful employment of thiophosphoryl nucleoside triphosphates for incorporation into DNA or to protect DNA from hydrolytic degradation is obviously dependent on the properties of the specific polymerase or exonuclease used.

A final important difference between the two polymerase enzymes is manifested by the inability of the T4 enzyme to carry out extensive polymerization on a poly[d(A-T)] template when both dATPαS and TTPαS are employed as substrates. In contrast Pol I or the Klenow fragment functions normally. Whether this anomalous behavior extends to other template-primers and eventually affects Pol I activity as well is at present undefined.

ACKNOWLEDGMENT

This work was supported in part by an NIH grant, GM 13306, to SJB.

REFERENCES

1. Kornberg, A. (1980) DNA Replication (Freeman, San Francisco).
2. Hall, Z.W., and Lehman, I.R. (1968) J. Mol. Biol. 36, 321-333.
3. Loeb, L.A., Dube, D.K., Beckman, R.A., Koplitz, M., and Gopinathan, K.P. (1981) J. Biol. Chem. 256, 3978-3987.
4. Gupta, A., DeBrosse, C., and Benkovic, S. (1982) J. Biol. Chem. 257, 7689-7692.
5. Das, S.K., and Fujimura, R.K. (1980) J. Biol. Chem. 255, 7149-7154.
6. Brutlag, D., and Kornberg, A. (1972) J. Biol. Chem. 247, 241-248.
7. Muzyczka, N., Roland, R.L., and Bessman, M.J. (1972) J. Biol. Chem. 247, 7116-7122.
8. Bessman, M.J., Muzyczka, N., Goodman, M.F., and Schnaar, R.L. (1974) J. Mol. Biol. 88, 409-421.
9. Hershfield, M.S. (1973) J. Biol. Chem. 248, 1417-1423.

10. Galas, D.J., and Branscomb, E.W. (1978) J. Mol. Biol. 124, 653-687.
11. Goodman, M.F., Hopkins, R., and Gore, W.C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4806-4810.
12. Clayton, L.K., Goodman, M.F., Branscomb, E.W., and Galas, D.J. (1979) J. Biol. Chem. 254, 1902-1912.
13. Setlow, P., Brutlag, D. and Kornberg, A. (1972) J. Biol. Chem. 247, 224-231.
14. Murray, A.W., and Atkinson, M.R. (1968) Biochem. 7, 4023-4029.
15. Chen, J.T., and Benkovic, S.J. (1983) Nucleic Acid Res. 11, 3737-3751.
16. Modrich, P., and Lehman, I.R. (1970) J. Biol. Chem. 245, 3226-3231.
17. Richardson, C.C., Schildkraut, C.L., Aposhian, H.V., and Kornberg, A. (1964) J. Biol. Chem. 239, 222-232.
18. Das, S.K., and Fujimura, R.K. (1980) Nucleic Acid Res. 8, 657-671.
19. Hershfield, M.S., and Nossal, N.G. (1972) J. Biol. Chem. 247, 3393-3404.
20. Englund, P.T. (1971) J. Biol. Chem. 246, 3269-3276.
21. Price, S.S., Schwing, J.M., and Englund, P.T. (1973) J. Biol. Chem. 248, 7001-7006.
22. Romaniuk, P.J., and Eckstein, F. (1982) J. Biol. Chem. 257, 7684-7688.
23. Burgers, P.M.J., and Eckstein, F. (1979) Biochem. 18, 592-596.
24. Thomas, K.R., and Olivera, B.M. (1978) J. Biol. Chem. 253, 424-429.
25. Benkovic, S.J., and Schray, K.J. (1971) The Enzymes 8, 201.
26. Gillin, F.D., and Nossal, N.G. (1976) J. Biol. Chem. 251, 5225-5232.
27. Kunkel, T.A., Eckstein, F., Mildvan, A.S., Koplitz, R.M., and Loeb, L.A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6734-6738.
28. Putney, S.D., Benkovic, S.J., and Schimmel, P.R. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7350-7354.
29. Goodman, M.F., Gore, W.C., Muzyczka, N., Bessman, M.J. (1974) J. Mol. Biol. 88, 423-435.