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Rate-Limiting Steps in the DNA Polymerase I Reaction Pathway†

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ABSTRACT: The initial rates of incorporation of dTTP and thymidine 5′-*O*-(3-thiotriphosphate) (dTTPαS) into poly(dA)·oligo(dT) during template-directed synthesis by the large fragment of DNA polymerase I have been measured by using a rapid-quench technique. The rates were initially equal, indicating a non-rate-limiting chemical step. However, the rate of thionucleotide incorporation steadily diminished to 10% of its initial value as the number of consecutive dTMPαS residues in the primer strand increased. This anomalous behavior can be attributed to the helix instability inherent in phosphorothioate-containing duplexes. Positional isotope exchange experiments employing the labeled substrate [α-$^{18}O_2$]dATP have revealed negligible α,β-bridging → β-nonbridging isotope exchange in template-directed reactions of *Escherichia coli* DNA polymerase I (Pol I) both in the presence and in the absence of added inorganic pyrophosphate (PP_i), suggesting rapid PP_i release following the chemical step. These observations are consistent with a rate-limiting step that is tentatively assigned to a conformational change of the E·DNA·dNTP complex immediately preceding the chemical step. In addition, the substrate analogue (S_P)-dATPαS has been employed to examine the mechanism of the PP_i exchange reaction catalyzed by Pol I. The net retention of configuration at the α-P is interpreted in terms of two consecutive inversion reactions, namely, 3′-hydroxyl attack, followed by PP_i attack on the newly formed primer terminus. Kinetic analysis has revealed that while α-phosphorothioate substitution has no effect upon the initial rate of polymerization, it does attenuate the PP_i exchange reaction by a factor of 15–18-fold. The mechanistic implications of these results are discussed.

Escherichia coli DNA polymerase I (Pol I)[1] is a relatively well-characterized multifunctional enzyme involved in the repair and replication of DNA in vivo. In addition to the polymerase capacity, which catalyzes the incorporation of mononucleotide residues derived from deoxyribonucleoside 5′-triphosphates (dNTPs) into the 3′ end of an appropriate template-primer, the enzyme is also capable of catalyzing DNA degradation by means of distinct 3′→5′- and 5′→3′-

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; KF, Klenow fragment; PP_i, inorganic pyrophosphate; (●), ^{18}O; kDa, kilodalton(s); PEP, phosphoenolpyruvate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PIX, positional isotope exchange; TEAB, triethylammonium bicarbonate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; GC–MS, gas chromatography–mass spectroscopy; b, bridging; nb, nonbridging; EDTA, ethylenediaminetetraacetic acid; dTTPαS, thymidine 5′-*O*-(3-thiotriphosphate); dTMPS, 2′-thymidine 5′-*O*-phosphorothioate.

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exonucleolytic activities, as well as by net pyrophosphorolysis. The 3′→5′-exonuclease activity is generally believed to be responsible for "proof-reading" the elongating primer chain, thereby ensuring high-fidelity replication in accordance with the Watson–Crick base-pairing rules (Kornberg, 1980). The dNTP → dNMP pool "turnover" and the PP_i exchange reactions catalyzed by Pol I are a consequence of the coupling of the polymerization reaction with the 3′ → 5′ hydrolysis and the pyrophosphorolysis reactions, respectively.

Pol I, which has been shown to consist of a single 103-kDa polypeptide chain (Jovin et al., 1969), can undergo limited proteolysis to yield two fragments: a large (Klenow) fragment (KF, 68 kDa) that retains the polymerase and 3′→5′-exonuclease activities and a small fragment (36 kDa) that retains the 5′→3′-exonuclease activity (Kornberg, 1980).

Considerable attention has recently been focused upon certain of the Pol I catalyzed reactions by means of a variety of stereochemical and kinetic analyses. The observations that both the polymerization and the 3′ → 5′ hydrolysis reactions proceed with net inversion of configuration at phosphorus have been taken as evidence arguing against the existence of nucleotidyl-enzyme intermediates along the associated reaction pathways (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984). The polymerase activity has been the subject of a recent detailed investigation by Bryant et al. (1983). Their pre-steady-state kinetic analysis of the polymerization reaction utilizing the homopolymer template-primer system $(dA)_{1000}$·$(dT)_{10}$ suggested the existence of two rate-limiting steps of approximately equal magnitude along the polymerization pathway, which were ascribed to first-order processes immediately preceding and following the nucleophilic substitution step.

In the present study, the nature of the nucleophilic substitution step in the polymerization cycle has been further investigated, primarily by means of analyzing the effects of substitution of α-phosphorothioate deoxyribonucleoside triphosphate (dNTPαS) substrates upon the pre-steady-state polymerization kinetics. In addition, the role of the PP_i release step in this cycle has been studied by means of positional isotope exchange (PIX) analysis, and finally, dNTPαS substrates have been employed to establish the stereochemistry and to examine the kinetics of the PP_i exchange reaction catalyzed by Pol I.

Experimental Procedures

Materials

DNA polymerase I was purified from both *E. coli* 594 (λ *Pol* AcI857 *nin* 5 *Q am* 73 *Sam* 7) and *E. coli* 964 (λ *Pol* $A^1att^+red^+imm^{\lambda}cI857$ *nin* 5 *Q am* 73 *Sam* 7) according to a published procedure (Davis et al., 1980). The *E. coli* strains were kindly furnished by Professor R. Davis and Professor A. Kornberg, respectively. The Klenow fragment was purified from *E. coli* CJ155 according to Joyce & Grindley (1983). The *E. coli* strain was kindly provided by Professor C. Joyce. Polyacrylamide gel electrophoresis of the purified enzymes indicated at least 95% homogeneity. Protein concentrations were determined spectrophotometrically using extinction coefficients of 9.26×10^4 M^{-1} cm^{-1} at 280 nm for Pol I (Jovin et al., 1969) and 6.32×10^4 M^{-1} cm^{-1} at 278 nm for KF (Setlow et al., 1972).

Adenylate kinase, pyruvate kinase, and apyrase were from Sigma Chemical Co. $(dA)_{1000}$, $(dT)_{10}$, poly(dT)·$(dA)_{12-18}$, and poly[d(A-T)] were from P-L Biochemicals. [^{3}H]dTTP and [^{3}H]dATP were from ICN Radiochemicals. $Na_4[^{32}P]PP_i$ and (S_P)-[^{35}S]dTTPαS were from New England Nuclear. $H_2{}^{18}O$ (99%) was from KOR Isotopes; dTTP, dATP, ATP, and PEP were from Sigma Chemical Co. dATPαS (S_P and R_P) were prepared as previously described (Gupta et al., 1982). (S_P)-dTTPαS was kindly furnished by Dr. J. T. Chen of this laboratory.

Liquiscint liquid scintillation cocktail was from National Diagnostics. Triethyl phosphate, pyridine, and triethylamine were distilled from CaH_2 immediately before use. $PSCl_3$ and $POCl_3$ were freshly distilled before use. DE81 and GF/C (2.5-cm) filters were from Whatman. Alkaline Norit-A was from Fischer Scientific Co. DEAE-Sephadex A-25 was from Pharmacia Fine Chemicals. Dowex AG1-X8 (100–200 mesh) was from Bio-Rad. DE-52 cellulose was from Whatman. TLC was performed on poly(ethylenimine) plates (Merck), developing with 1 M KCl. Thiophosphate-containing compounds were visualized with 1% 2,6-dibromobenzoquinone 4-chloroimine (Eastman) in glacial acetic acid. Reverse-phase HPLC was performed on an Altex 100 system equipped with a 25 × 0.46 cm Whatman ODS-2 column, eluting isocratically with 40 mM potassium phosphate, pH 6.0, at 1.5 mL/min. Retention times for dATP, (S_P)-dATPαS, and (R_P)-dATPαS were 6.6, 10.0, and 13.6 min, respectively.

Methods

DNase I Activation of Poly[d(A-T)]. The procedure of Modrich & Lehman (1970) was used to prepare poly[d(A-T)] of average chain length 1000 nucleotides, as measured by Pol I catalyzed 3′ end labeling with [^{3}H]dTMP.

Polymerase Assays. Polymerization time courses were followed either by the DE81 filter paper assay (Bryant et al., 1983) or by collection of acid-precipitable radioactivity on a GF/C filter (Deutscher & Kornberg, 1969). Filters were counted by liquid scintillation counting in 5 mL of a standard toluene-based scintillation fluid. Reaction mixtures were as indicated in the specific experiments and/or in the figure legends.

PP_i Exchange Assays. The method of Seal & Loeb (1976) for measuring the conversion of $[^{32}P]PP_i$ into a Norit-adsorbable form was employed, with the following modifications. The 10% Norit suspension was prepared in 100 mM Na_4PP_i, which greatly reduced nonspecific adsorption of $[^{32}P]PP_i$. After filtration, the Norit was washed with 50 mL of 6 mM HCl, and the radioactivity collected on the GF/C filter was counted by liquid scintillation counting in 10 mL of Liquiscint.

Preparation of [α-$^{18}O_2$]dATP. Deoxyadenosine monohydrate (0.515 mmol) was dried by repeated evaporation from anydrous pyridine, dissolved in 3 mL of triethyl phosphate, and cooled on ice. Freshly distilled $POCl_3$ (0.536 mmol) was added dropwise and the solution stirred on ice for 1.5 h. $H_2{}^{18}O$ (150 μL) was added and the mixture stirred at 4 °C for 1.3 h before the addition of 1 mL of anhydrous triethylamine and further stirring for 3 h. Water (1.5 mL) was added, and the solution was kept overnight at 4 °C. Dilution to 50 mL with water followed by chromatography over a DE-52 cellulose column (3.0 × 18.0 cm), eluting with a 1.5-L linear gradient of 0–0.2 M TEAB, yielded [$^{18}O_2$]dAMP (0.256 mmol, 50%).

A 3-mL reaction containing 100 mM Tris·HCl (pH 8.0), 25 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol 0.5 mM ATP, 62 mM PEP, 25 mM [$^{18}O_2$]dAMP, 300 units of adenylate kinase, and 242 units of pyruvate kinase was incubated at 22 °C for 20 h. The mixture was diluted to 10 mL and chromatographed over a DEAE-Sephadex A-25 column (1.5 × 22.5 cm), eluting with a 1-L linear gradient of 0.1–0.8 M TEAB. Fractions (15 mL) were collected and monitored spectrophotometrically at 260 nm. [α-$^{18}O_2$]dATP eluted at ca. 0.47 M TEAB in 60% yield.

^{31}P NMR analysis (D_2O, pH 8.8) of the α-P revealed four species, consistent with a mixture of only singly and doubly labeled material. Integration of the peak areas gave the following distribution of products: $^{18}O_2$ (nb,nb), 20%; $^{18}O_2$ (b,nb), 42%; ^{18}O (nb), 14%; ^{18}O (b), 24% ± 2%. In addition, the small extent of apyrase-directed attack at the α-P offered a convenient means of evaluating the isotopic enrichment of the ^{18}O-labeled dATP. A 0.71-μmol fraction of the labeled material was treated with 1 unit of apyrase for 1 h, as described below for the PIX experiments. GC–MS analysis of the P_i (as trimethyl phosphate) thus isolated gave an average m/e 144/142 ratio of 1.66 ($Me_3P^{18}O_2O_2/Me_3P^{18}OO_3$), indicating 62.3% [$\alpha$-$^{18}O_2$]dATP and 37.7% [$\alpha$-$^{18}O$]dATP, in good agreement with the above NMR analysis.

Rapid-Quench Experiments. Rapid-quench time points between 10 ms and 10 s were obtained by using a quench-flow apparatus designed and constructed in this laboratory. This apparatus will be described in more detail at a later date (K. A. Johnson, unpublished results). Briefly, 20-μL samples were loaded into segments of tubing using three-way Hamilton valves. The position of the three-way valve was then changed to allow drive syringes containing buffer to force the reactants through a T mixer and then through a variable-length segment of tubing into a quench solution. The length of tubing leading to the quench solution was varied to obtain time points from 10 to 100 ms. The syringes containing buffer were driven by using a ball screw and a stepping motor as described by Froelich et al. (1976). The programmable stepping motor provided precisely controlled movements of the drive syringes including essentially instantaneous start/stop at full speed (1000 steps per second). Reaction times longer than 100 ms were obtained by driving the syringes once to force the mixing of reactants, pausing for a defined interval of time, and then driving the syringes a second time to quench the reaction.

The desired temperature was maintained with a circulating water bath. Reactions were initiated by mixing equal volumes (20 μL) of the enzyme–polymer and Mg–[^{3}H]dTTP or Mg–[^{35}S]dTTPαS solutions and were quenched into 50 μL of 0.1 M EDTA contained in a sealed siliconized 1.5-mL polyethylene microtube. The stock enzyme–polymer solutions contained 400 nM poly(dA)·oligo(dT) (1/1 polymer/oligomer) and 65.4 nM KF in 50 mM Tris·HCl (pH 7.4) and 800 nM poly(dA)·oligo(dT), 196 nM KF, and 4 mM dithiothreitol in 50 mM Tris·HCl (pH 7.4) for the dTTP and the dTTPαS reactions, respectively. The stock Mg–dTTP/dTTPαS solutions contained 4 mM $MgCl_2$ and 66 μM [^{3}H]dTTP or [^{35}S]dTTPαS (3000–4500 cpm/pmol) in 50 mM Tris·HCl (pH 7.4). The inclusion of dithiothreitol in the thiophosphate assay was found to greatly reduce the extent of nonspecific binding of [^{35}S]dTTPαS to DE81 paper, thus reducing the background radioactivity without affecting the polymerization rate.

Zero-time points were obtained manually in triplicate by the addition of 20 μL of the stock Mg–dTTP/dTTPαS solution to 50 μL of 0.1 M EDTA prior to the addition of 20 μL of the stock enzyme–polymer solution. All other time points were obtained in duplicate. The ca. 90 μL of quenched solution collected per time point was assayed for total radioactivity by applying 5 μL to a GF/C filter, which was air-dried and counted in 5 mL of toluene-based liquid scintillation fluid. An 80-μL aliquot of the remaining solution was assayed for the incorporation of [^{3}H]dTMP or [^{35}S]dTMPS into polymeric form by the DE81 filter assay (Bryant et al., 1983).

The steady-state rate of [^{3}H]dTMP incorporation measured by the rapid-quench technique was in close agreement with the steady-state rate measured manually under identical conditions (22 °C), thus arguing against DNA shearing upon rapid mixing.

Processivity Measurements. The processivities of Pol I and KF in the replication of poly(dA)·oligo(dT) utilizing either dTTP or dTTPαS as the polymerization substrate were estimated by the E·polymer trapping method of Bryant et al. (1983). Reaction solutions contained 25 nM Pol I or KF, 200 nM poly(dA)·oligo(dT), 2 mM $MgCl_2$, 33 μM [^{3}H]dTTP or [^{35}S]dTTPαS (1000–2000 cpm/pmol), and 325 μg/mL heat-denatured calf thymus DNA (where applicable) in 50 mM Tris·HCl (pH 7.4). Reactions were quenched after selected time intervals with EDTA to a concentration of 40 mM and analyzed for incorporation of radioactivity into polymer by the DE81 filter paper assay.

PIX Experiments. PIX reactions (15 mL) containing 67 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, 6.7 mM $MgCl_2$, 50–100 μM poly[d(A-T)] or poly(dT)·$(dA)_{12-18}$ (total nucleotide), 50 μM dNTPs, and 0–2 mM Na_4PP_i were initiated with Pol I to a concentration of 7 nM and were incubated at 37 °C. Polymerization and pyrophosphate exchange were monitored, where applicable, by supplementing 0.3-mL aliquots of the reaction mixture with either [^{3}H]dATP or [^{32}P]PP_i prior to initiation and assaying 50-μL aliquots at given times as described above. The reactions were quenched with EDTA to a concentration of 50 mM after 1–5 h and were chromatographed over DEAE-Sephadex A-25 columns (1.5 × 24.0 cm) eluting with a 1-L linear gradient of 0.2–0.5 M TEAB. The resolved dNTPs recovered by evaporation were treated with apyrase to remove the β- and γ-phosphate residues. Apyrase reaction mixtures (0.5 mL) containing 50 mM sodium succinate (pH 6.5), 5 mM $CaCl_2$, and 1 unit of apyrase were incubated at 22 °C for 1 h (Walseth et al., 1981). After being quenched on ice, the reactions were brought to pH 8.0 with Tris base and chromatographed over Dowex AG1-X8 columns (1.0 × 4.0 cm), eluting first with 10 mM HCl and then with 30 mM HCl (Hackney et al., 1980). Fractions (2 mL) were collected and assayed for P_i by the colorimetric assay of Lanzetta et al. (1979).

The P_i-containing fractions were pooled and lyophilized. The residue was dissolved in methanol and methylated with excess ethereal diazomethane. The resulting trimethyl phosphate was analyzed by GC–MS as previously described (Sharp & Benkovic, 1979). All GC–MS runs were performed in duplicate. Integrated GC–MS peaks (m/e 140, 142, 144) were corrected for the natural abundance of ^{18}O, experimentally determined by a blank run to be 0.278%. Integrated values for m/e 142 ($Me_3P^{18}OO_3$) were also corrected for the small extent of apyrase-catalyzed hydrolysis that occurs at the α-P of the triphosphate under the reaction conditions employed, using the average m/e 144/142 ratio of 1.66 reported above and the natural abundance corrected intensity of the m/e 144 ($Me_3P^{18}O_2O_2$) peak. In addition, the m/e 142 values were corrected, where applicable, for PP_i exchange, the extent of which was measured as described above.

Stereochemistry of PP_i Exchange. Reactions containing 50 mM Tris·HCl (pH 8.0), 1.5 mM dithiothreitol, 6.7 mM $MgCl_2$, 150 μM poly[d(A-T)] or poly(dT)·$(dA)_{12-18}$, 1.5 mM Na_4[^{32}P]PP_i (35–40 cpm/pmol), and 150 μM (S_P)-dATPαS or (R_P)-dATPαS in 0.5 mL were initiated by the addition of Pol I to a concentration of 25 nM and were incubated at 37 °C. Aliquots (40 μL) were withdrawn after 0.5 and 1 h [poly[d(A-T)] reaction], or after 1 and 2 h [poly(dT)·$(dA)_{12-18}$ reaction], and assayed for Norit-adsorbable radioactivity. Reactions were quenched by the addition of EDTA to a

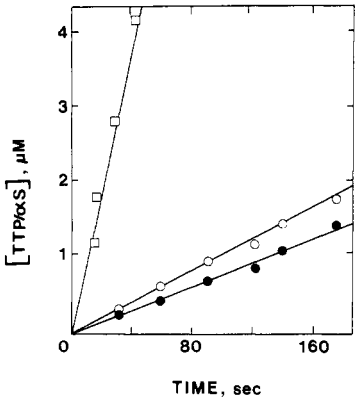


FIGURE 1: Comparative steady-state polymerization time courses (22 °C). The final reaction solutions contained 190 nM Klenow fragment, 25 nM poly(dA)·oligo(dT), 2 mM $MgCl_2$, and 33 μM [^{3}H]dTTP (□), 33 μM [^{35}S]dTTPαS (●), or 100 μM [^{35}S]dTTPαS (○) in 50 mM Tris·HCl (pH 7.4).

concentration of 40 mM and were then treated with 60 nmol each of cold carrier dATP, (S_P)-dATPαS, and (R_P)-dATPαS and chromatographed over DEAE-Sephadex A-25 columns (0.6 × 27.0 cm) eluting with a 250-mL linear gradient of 0.1–0.8 M TEAB. Spectrophotometrically monitored column elutions (260 nm) were collected in 2-mL fractions. The radioactivity was located by counting 0.1-mL aliquots of the appropriate fractions in 3 mL of Liquiscint. Radioactivity-associated dATPαS fractions (not resolved into diastereomers on this column) were only isolated from those incubation mixtures initially containing (S_P)-dATPαS. The (R_P)-dATPαS reactions either showed no incorporation of ^{32}P [poly(dT)·$(dA)_{12-18}$ reaction] or showed incorporation only into the dATP contaminant [poly[d(A-T)] reaction]. The lack of ^{32}P incorporation in the former case was not due to enzyme inactivity, as demonstrated by the formation of Norit-adsorbable radioactivity in a 40-μL aliquot of the 2-h incubation mixture following addition of dATP and reincubation at 37 °C for 4 min.

The exchanged dATPαS fractions were pooled and concentrated to 0.2 mL. The configuration of the exchanged dATPαS was determined by HPLC analysis of 0.1-mL samples. A fraction collector was attached beyond the UV detector. The A_{260} of each 2-mL fraction was measured and the entire fraction subsequently counted in 15 mL of Liquiscint. The radioactivity was observed to coelute exclusively with authentic (S_P)-dATPαS.

RESULTS

Polymerization Kinetics Using a Thionucleotide Substrate. As illustrated in Figure 1, a substantial retardation of the polymerization rates of poly(dA)·oligo(dT), catalyzed by both Pol I and KF, is observed upon replacement of dTTP by (S_P)-dTTPαS as the nucleotide substrate. The polymerization reactions show slightly different triphosphate concentration dependencies: inhibition is observed at [dTTP] > 33 μM, whereas the polymerization rate is found to increase by ca. 25% over the 33–100 μM [dTTPαS] range. An analogous effect was observed on the reaction catalyzed by Pol I.

The substrate substitution effect on the steady-state polymerization rate of poly(dA)·oligo(dT) thus differs from the mere 20% initial rate attenuation found upon replacement of dATP by (S_P)-dATPαS in the Pol I catalyzed replication of the alternating copolymer poly[d(A-T)] (Burgers & Eckstein, 1979; Gupta et al., 1984). In order to identify the factor that is responsible for the 10–15-fold rate difference depicted by Figure 1, the pre-steady-state kinetics of the KF-catalyzed

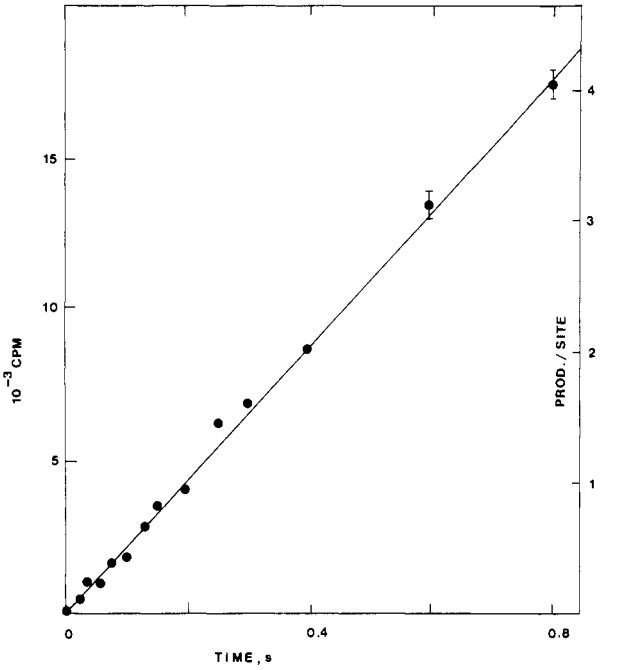


FIGURE 2: Rapid-quench polymerization time course of [^{3}H]dTMP incorporation into poly(dA)·oligo(dT) catalyzed by the Klenow fragment (22 °C). Reactions were initiated by the addition of a solution containing 50 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, and 66 μM [^{3}H]dTTP to an equal volume of a preincubation mixture containing 50 mM Tris·HCl (pH 7.4), 400 nM poly(dA)·oligo(dT), and 65.4 nM Klenow fragment. Product/site denotes the number of nucleotide residues incorporated per enzyme.

incorporation of dTMP and dTMPS into poly(dA)·oligo(dT) were studied by means of a rapid-quench technique. The respective polymerization time courses are illustrated in Figures 2 and 3. Relatively high background radioactivity in the thiophosphate assay necessitated amplification of the incorporation levels by employing higher enzyme and DNA concentrations.

The incorporation data were initially fit to the same model as that described by Bryant et al. (1983) (eq 1) where E

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E \qquad (1)$$

represents the E·DNA complex, S is MgTTP/TTPαS, and P is the elongated primer. The absence of a pre-steady-state burst in the dTTP time course (Figure 2) indicates the existence of a single rate-limiting step (k_2) in the polymerization pathway. In contrast, the curvature apparent is the dTTPαS time course (Figure 3) cannot be simply explained in terms of rate-limiting product release (k_3) for which a maximum burst amplitude[2] corresponding to the incorporation of one product for site would be expected. The time course instead indicates a progressively diminishing rate of incorporation as the number of successive dTMPαS residues in the primer strand increases. For comparative purposes, the steady-state rate of incorporaton of [^{3}H]dTMP was measured manually under conditions identical with those described in Figure 3. The rate was found to be in close agreement with the initial

[2] The transient incorporation of nucleotide into polymer as predicted by this model is given by the rate equation (Gutfreund, 1972):

$$[P] = [ES]\left\{\frac{k_2k_3}{k_2 + k_3}t + \left(\frac{k_2}{k_2 + k_3}\right)^2[1 - e^{-(k_2+k_3)t}]\right\}$$

where the steady-state rate $k_{cat} = k_2k_3/(k_2 + k_3)$ and the burst amplitude $= [k_2/(k_2 + k_3)]^2[ES]$.

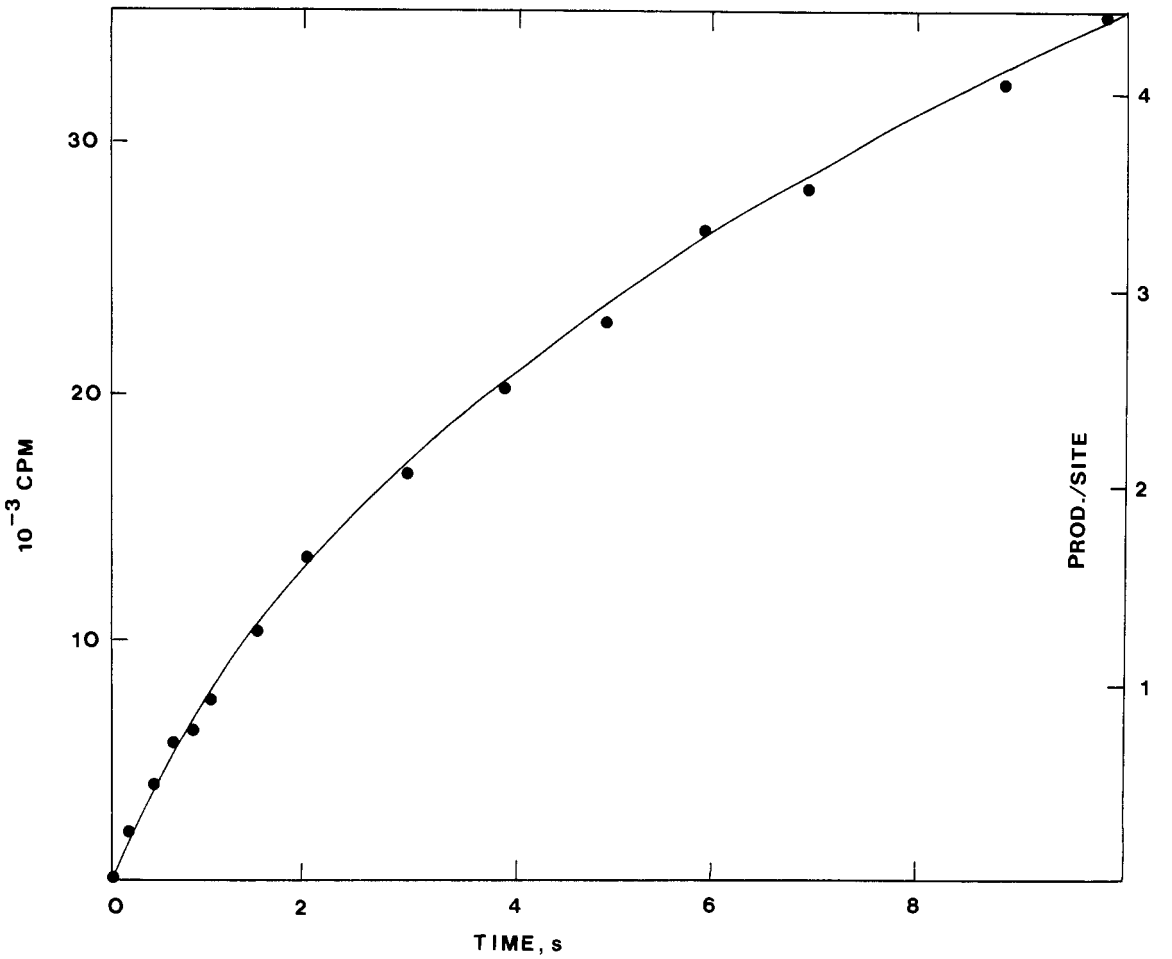


FIGURE 3: Rapid-quench polymerization time course of [^{35}S]dTMPS incorporation into poly(dA)·oligo(dT) catalyzed by the Klenow fragment (22 °C). Reactions were initiated by the addition of a solution containing 50 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, and 66 μM [^{35}S]dTTPαS to an equal volume of a preincubation mixture containing 50 mM Tris·HCl (pH 7.4), 4 mM dithiothreitol, 800 nM poly(dA)·oligo(dT), and 196.4 nM Klenow fragment.

rate of dTMPS incorporation ($k^{PO} = 1.6k^{PS}$). Since the initial and steady-state rates of KF-catalyzed incorporation are identical (Figure 2), chemical bond formation can be convincingly eliminated as the rate-limiting step in the polymerization cycle.

Recent studies of the effects of the thiophosphodiester linkages upon the properties of poly[d(A-^{s}T)] and poly[d(T-^{s}A)] have revealed a significantly lower melting temperature in both cases relative to poly[d(A-T)], thus implicating helix instability due to phosphorothioate substitution in DNAs (Eckstein & Jovin, 1983). It could be argued that the helix destabilization effect is more pronounced in the polymerization product of poly(dA)·oligo(dT) with dTTPαS than in the alternating copolymer formed with poly[d(A-T)] by using either dATPαS or dTTPαS, accounting for the apparent discrepancy in the substitution effects on the steady-state kinetics of the two systems. The enhanced sensitivity toward the altered secondary structure of the homopolymer template-primer is reflected by the curvature in the dTTPαS rapid-quench time course (Figure 3).

In addition, the effect of phosphorothioate substitution upon the processivities of Pol I and KF has been investigated. Table I summarizes the results obtained. According to the model proposed by Bryant et al. (1983), the processivity of a polymerase can be described in terms of a partitioning of the E–DNA complex between polymerization (k_{cat}) and dissociation (k^{ss}_{off}). Processivities were measured by the polymer-trapping method of Bryant et al. (1983). The negligible

Table I: Effect of Substrate Substitution upon the Processivities of Pol I and KF (22 °C)

enzyme	$k_{cat}^{PO}/k_{cat}^{PS}$	processivity[a]	
		dTTP	dTTPαS
Pol I	12	45–50[b]	<4
KF	10	20–25	<4

[a] The number of nucleotide residues incorporated per encounter of enzyme with template-primer, measured as described under Methods. [b] From the data of Bryant et al. (1983).

processivity of both polymerases on the poly(dA)·oligo(dT) template-primer with dTTPαS as the triphosphate substrate can be accounted for in terms of the 10–12-fold reduction in k_{cat}.[3]

On the basis of the detection limits of the polymerization assay employed, the linearity of the KF-catalyzed dTTP incorporation time course (Figure 2) places the rate of product release or "translocation" (k_3, eq 1) at >4 times that of the earlier rate-limiting step (k_2). This situation differs from that observed for the analogous Pol I catalyzed cycle, for which $k_2 \simeq k_3$ (Bryant et al., 1983). These observations suggest an

[3] This model predicts the time dependence of the number of nucleotides incorporated per enzyme as follows:

$$\left(\frac{N}{E}\right)_t = \frac{k_{cat}}{k^{ss}_{off}}(1 - e^{-k^{ss}_{off}t})$$

where the "processivity", i.e., $(N/E)_{t\to\infty}$, is given by k_{cat}/k^{ss}_{off}.

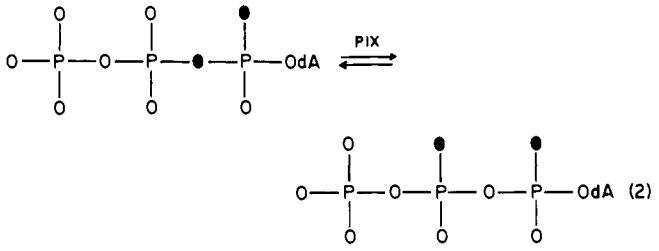
Table II: Mass Spectral Analysis of PIX Reactions of [α-$^{18}O_2$]dATP[a]

template-primer	concn (μM)[b]	dNTP	[PP_i] (mM)	% polymerized	% PP_i exchanged	% increase in *m/e* 142 integrated intensity[c,d]
poly[d(A-T)]	50	A	1.5	3.0	6.2	1.16
		T[e]				0.06
	90	A		23.0		0.23
		T				0.13
	100	A	1.5	6.0	45.0	0.48
		T				0.40
poly(dT)·$(dA)_{12-18}$	76	A	1.5	13.0	40	0.38
	90	A				0.88

[a] See Methods for experimental details. [b] Expressed as nucleotide concentration. [c] Obtained by correcting the measured *m/e* 142 integrated intensity for the contributions of 0.278% ^{18}O natural abundance, α-P apyrase attack, and PP_i exchange (where applicable) as described under Methods. [d] Average of duplicate runs. [e] In poly[d(A-T)] reactions, both labeled dATP and unlabeled dTTP were analyzed by the same apyrase degradation procedure.

intrinsic difference in the polymerization kinetics of the intact polymerase compared to its proteolytic fragment and perhaps suggest a weakening of the E–DNA interaction upon removal of a 36-kDa polypeptide portion of the enzyme and thus a faster translocation step. In accordance with this conclusion is the observation that KF is 2-fold less processive than Pol I in replicating poly(dA)·oligo(dT) (Table I).

Positional Isotope Exchange (PIX) Experiments. The isotopically labeled triphosphate substrate [α-$^{18}O_2$]dATP has been employed to investigate the possible occurrence of a Pol I catalyzed template-dependent positional isotope exchange reaction involving α,β-bridging → β-nonbridging ^{18}O exchange:



The analytical method employed to monitor the exchange reaction involved controlled apyrase-catalyzed degradation of the recovered triphosphate to dAMP and P_i, followed by derivatization of the P_i to trimethyl phosphate, which was subjected to a mass spectral isotopic enrichment analysis. Since isotopic exchange of the type depicted by eq 2 would manifest itself in the formation of $^{18}OP_i$ by apyrase treatment, integration of the (M + 2) mass spectral peak (*m/e* 142, $Me_3P^{18}OO_3$) offers a means of evaluating the extent to which exchange occurs during a given reaction. Of the total singly (37.7%) and doubly (62.3%) ^{18}O-labeled dATP present, only two-thirds of the [α-$^{18}O_2$]dATP (41.5%) and one-third of the [α-^{18}O]dATP (12.6%) bear the requisite α,β-bridging ^{18}O label for PIX observation, thus yielding a total of 54.1% observable fraction. Complete equilibration of the β-nonbridging oxygen atoms would result in redistribution of the α,β-bridging ^{18}O label, such that two-thirds of the label exchanges to the β-nonbridging position and one-third remains at the α,β-bridging position. The total fraction of dATP bearing a β-nonbridging ^{18}O label resulting from such a process is thus expected to be 36.07%.[4] Considering the 50% dilution factor introduced into the GC–MS analysis by the completely unlabeled γ-P, an 18.04% increase in the (M + 2) species would be anticipated under optimal exchange conditions.

[4] This fraction is comprised of 27.69% and 8.38% contributions from [α-$^{18}O_2$]dATP and [α-^{18}O]dATP, respectively (doubly/singly labeled [α-^{18}O]dATP ratio = 62.3/37.7).

The results of a series of PIX experiments conducted under a variety of reaction conditions are given in Table II. The corrected *m/e* 142 intensity increase of [^{18}O]dATP-derived trimethyl phosphate appears to be independent of the nature of the template-primer, the presence of added PP_i, and the extent to which the reaction is allowed to proceed. The observed *m/e* 142 increases (<1.16%) are probably within the experimental error of the analytical procedure. Comparison with the theoretical 18.04% intensity increase associated with complete exchange indicates that under the polymerization and PP_i exchange conditions considered (Table II), the extent of α,β-bridging → β-nonbridging PIX of [α-$^{18}O_2$]dATP is negligible. In principle, these results may be interpreted in terms of torsional constraint of the bound PP_i product of primer elongation, preventing equilibration of the β-nonbridging oxygen atoms. However, a more likely explanation is extremely rapid departure of PP_i from the E–DNA complex, which strongly commits the reaction to the forward direction. Consistent with this explanation is the lack of appearance of labeled PP_i in dTTP via transfer from [α-$^{18}O_2$]dATP when poly[d(A-T)] served as the template-primer.

Stereochemistry of PP_i Exchange. The mechanism of the PP_i exchange reaction catalyzed by Pol I has been proposed to involve the interception by PP_i of an intermediate along the polymerization reaction coordinate (Kornberg, 1980). The relative ease of attainment of this intermediate from the polymerization direction offers a possible explanation of the significantly faster rate of PP_i exchange than pyrophosphorolysis.

The complete absence of PP_i exchange in reactions containing (R_P)-dATPαS as the complementary triphosphate substrate provides direct evidence supporting the involvement of 3′-hydroxyl attack on the triphosphate in the overall exchange reaction. Although (R_P)-dNTPαS's bind weakly to the triphosphate site of Pol I ($K_i \sim 300$ μM; Bryant et al., 1983), they are not accepted as substrates for the polymerase activity (Burgers & Eckstein, 1979). In contrast, reactions containing (S_P)-dATPαS, which is an acceptable polymerization substrate, were found to exhibit PP_i exchange, thereby implicating primer elongation as an initial step essential to the exchange process.

These observations justify extrapolating the stereochemical course of the exchange reaction from the combined knowledge of the configuration of the exchanged α-thiotriphosphate and the stereochemistry of the polymerization reaction. Configurational assignment, based upon an HPLC separation of the dATPαS diastereomers, revealed the exclusive formation of β,γ-^{32}P-labeled (S_P)-dATPαS in both the poly[d(A-T)]- and poly(dT)·$(dA)_{12-18}$-dependent exchange reactions (Figure 4).

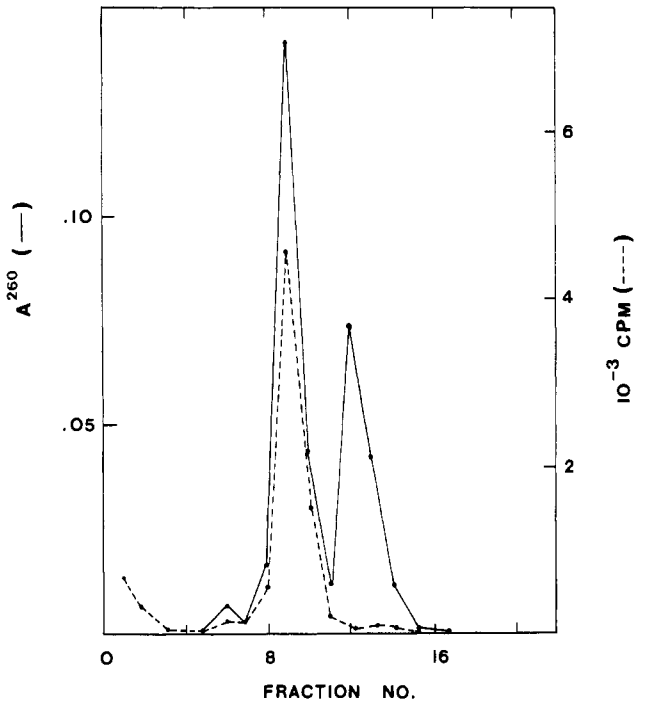


FIGURE 4: Stereochemistry of PP_i exchange. HPLC analysis of [^{32}P]PP_i-exchanged (S_P)-dATPαS catalyzed by Pol I on a poly-(dT)·$(dA)_{12-18}$ template-primer. Fractions were monitored spectrophotometrically (260 nm, —) and by liquid scintillation counting (---). (See Methods for details.)

Since the polymerization step proceeds with inversion of configuration (Burgers & Eckstein, 1979; Brody & Frey, 1981), PP_i attack on the elongated primer must also occur with inversion to account for the net retention observed in the exchange experiments.

Kinetics of PP_i Exchange. The effects of phosphorothioate substitution upon the kinetics of the PP_i exchange reaction have been investigated. Subtitution of only one of the triphosphate substrates by its α-thio analogue has little apparent effect on the initial rate of the poly[d(A-T)]-dependent PP_i exchange reaction. However, complete substitution reduces the exchange rate by 15–50-fold in both the poly[d(A-T)]- and poly-(dA)·oligo(dT)-dependent reactions. The comparative PP_i exchange rates were determined under conditions allowing net polymerization to occur, albeit at a reduced rate, owing to inhibition by PP_i (McClure & Jovin, 1975). As discussed above, an α-PO → α-PS substitution has no effect upon the initial polymerization rate. However, the data of Figure 3 suggest that phosphorothioate-induced helix instability does have a pronounced effect upon the polymerization rate using a homopolymer template-primer system. In addition, Gupta et al. (1984) have observed an analogous attenuation of the polymerization rate upon more extensive phosphorothioate substitution of the alternating copolymer poly[d(A-T)]. In view of the apparently strict base-paired primer terminus requirement of the PP_i exchange reaction (Kornberg, 1980), it is thus conceivable that the large effects of thio substitution on the PP_i exchange rate are solely attributable to the concomitant helix instability factor.

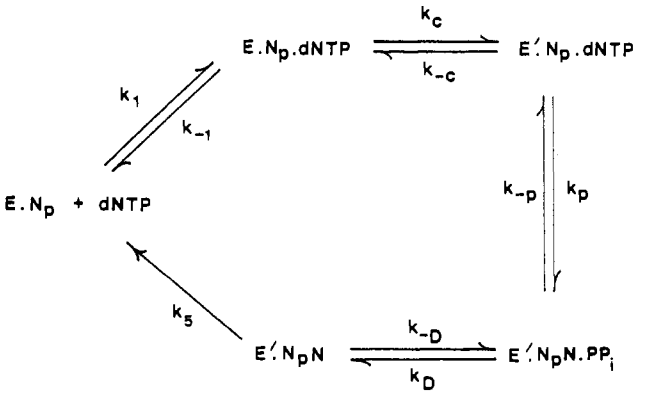
In order to minimize this problem, the substitution effect upon the poly[d(A-T)]-dependent PP_i exchange rate was determined for reactions containing only one triphosphate substrate (dTTP or dTTPαS). Under such conditions, PP_i exchange is indeed supported, albeit at a slightly slower rate (Table III), but net polymerization is precluded by the absence of the second complementary nucleotide substrate. As the

Table III: Effects of Phosphorothioate Substitution upon PP_i Exchange Rates (22 °C)[a]

template	dNTP/αS	k_{rel}
poly[d(A-T)]	A, T	100
	^{S}A, T	89 ± 10
	A, ^{S}T	90 ± 10
	^{S}A, ^{S}T	7 ± 2
	T	85 ± 10
	^{S}T	5 ± 1
poly(dA)·oligo(dT)	T	98
	^{S}T	3 ± 1

[a] Reaction mixtures (100 μL) contained 60 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, 6.6 mM $MgCl_2$, 1.5 mM [^{32}P]PP_i (100–120 cpm/pmol), 50 μM dNTP/αS (as specified), 8 nM KF, and either 150 μM poly[d(A-T)] (as nucleotide) or 2.5 μM poly(dA)·oligo(dT) (as 3′ ends, 1:50 polymer:oligomer). Aliquots (20 μL) were assayed for Norit-adsorbable radioactivity at selected times (0–30 min).

Scheme I



results presented in Table III indicate, a considerable elemental substitution effect (15–18-fold) nonetheless persists, despite the fact that the number of dNMPαS residues per primer strand has been limited to 1.

Discussion

The present study has been directed toward investigating both the role of the nucleophilic displacement step involved in primer elongation and the subsequent interaction of the extended primer with PP_i as part of the overall mechanism of action of DNA polymerase I. The use of elementally and isotopically substituted nucleotide substrates has been employed for this purpose.

The results presented above dictate the formulation of an extended kinetic scheme to describe the enzyme-catalyzed polymerization reaction (Scheme I). The absence of a substitution effect on the rate of polymerization as indicated by the rapid-quench data confirms the existence of a rate-limiting step that occurs between triphosphate binding to the binary E–DNA complex (k_1) and chemical bond formation (k_p). This step remains tentatively assigned to a conformational change of the ternary complex (k_c) to a form poised for catalysis (Bryant et al., 1983).[5] The failure to observe PIX within the isotopically labeled substrate [α-$^{18}O_2$]dATP is indicative of a strong commitment to the forward reaction, such that the rate of PP_i release (k_D) greatly exceeds that of the reverse chemical step (k_{-p}). In addition, the comparative rapid-quench time courses of the Pol I catalyzed and KF-catalyzed polymerization reactions suggest that the magnitude of the free energy barrier to translocation, and initiation of the next polymerization cycle, is dependent on the enzyme concerned, with

[5] The use of fluorescently labeled enzyme as a means of resolving this problem is currently under investigation in this laboratory.

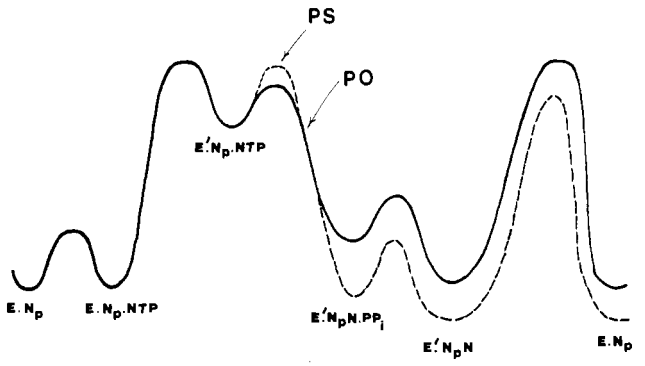


FIGURE 5: Energy profile of the reaction catalyzed by DNA polymerase I.

the barrier faced by Pol I apparently exceeding that faced by its large fragment. Assuming that k_p is at most partially rate limiting and that $k_D >> k_c$, the polymerization rate expression based on Scheme I at saturating [dNTP] and in the absence of added PP_i can be shown to simplify to

$$k_{Pol} = k_c k_5/(k_c + k_5)$$

Accordingly, if $k_5 >> k_c$, as is the case for KF, k_{Pol} is solely limited by the rate of the substitution-insensitive conformational change of the ternary complex (k_c).

The experimental observation that remains to be addressed is the 15–18-fold α-PO → α-PS substitution effect upon the rate of PP_i exchange, measured under conditions designed to minimize the potentially complicating helix instability factor associated with phosphorothioate-containing duplexes. According to Scheme I, the PP_i exchange pathway is represented by th reverse of the polymerization path up to and including the chemical step. On this basis, the PP_i exchange rate expression at saturating [PP_i] can be shown to simplify to the following, assuming $k_D >> k_{-p}$, $k_{-1} >> k_c$, and $k_p >> k_{-c}$:

$$k_{ex} = \frac{k_{-c}k_{-p}k_c}{k_{-c}k_{-p} + k_c k_{-p} + k_c k_p} = \frac{k_c}{1 + K_c + K_c K_p}$$

The equilibrium constants of the conformational change and the chemical step, K_c and K_p, respectively, are thus implicated in the simplified rate expression for PP_i exchange. As suggested above, the kinetics of the conformational change may well be substitution insensitive, i.e., $K_c^{PO} \simeq K_c^{PS}$. In contrast, the forward and reverse chemical steps are $S_N2(P)$ displacement reactions involving a secondary alcohol (3′-hydroxyl) and PP_i as the respective nucleophiles and, as such, are substitution sensitive. The lower reactivity (30–100-fold) of thiophosphodiesters than their phosphodiester analogues in bimolecular nucleophilic displacement reactions is well documented for chemical systems and has been attributed to the relatively weak inherent electrophilicity of a thiophosphoryl center (Benkovic & Schray, 1971). The precise magnitude of a PO → PS substitution effect is expected to be a sensitive function of the nature of both the nucleophile and the leaving group, and as such, it is plausible that $K_p^{PO} < K_p^{PS}$; i.e., the reverse chemical step is more substitution sensitive than the forward step. This situation is illustrated by the free energy diagram of Figure 5. The relative ground-state stabilization of E′·N_p(S)N·PP_i (and subsequent intermediates along the reaction coordinate) thus offers a means of accommodating the 15-18-fold substitution effect on the rate of PP_i exchange, while remaining consistent with the lack of an effect on the rate of polymerization.

The perhaps oversimplified representation of the "translocation" step as a single first-order process (k_5) is acknowledged. In accordance with the "next nucleotide" effects described by Fersht (1979) and by Kunkel et al. (1981), translocation of the enzyme may involve a triphosphate dependence, which may in turn assist in rationalizing the significantly faster rate of PP_i exchange than pyrophosphorolysis (Deutscher & Kornberg, 1969). Experiments bearing on this problem are currently under way in this laboratory.

The ability of (S_P)-dNTPαS substrates to undergo PP_i exchange, albeit at a reduced rate, has been exploited for the purpose of deducing the stereochemical course of the exchange reaction. The reaction has been found to proceed with net retention of configuration at phosphorus. This observation is most simply accounted for by two consecutive in-line displacements, namely, polymerization (Burgers & Eckstein, 1979; Brody & Frey, 1981) followed by PP_i attack at the elongated primer terminus and implies that PP_i exchange is on the polymerization reaction pathway. This in turn lends support to the discussion presented above concerning the effects of elemental substitution upon the kinetics of PP_i exchange. In conjunction with the stereochemical path of the 3′→5′-exonuclease reaction, which is also one of inversion (Gupta & Benkovic, 1984), this result further supports the argument against the existence of covalent nucleotidyl-enzyme intermediates along the associated pathways.

In conclusion, this study has demonstrated that due caution is required in drawing mechanistic conclusions on the basis of α-phosphorothioate substitution within the nucleotide substrate of an enzyme such as DNA polymerase I, largely as a result of the subtle, yet poorly characterized structural changes effected in the reaction product (Eckstein & Jovin, 1983). Such problems notwithstanding, the combination of elementally and isotopically substituted nucleotide substrates employed has provided a useful means of addressing kinetic and stereochemical aspects of the mechanism of action of Pol I. The kinetic description emerging from this and previous studies is one in which the E·DNA·dNTP ternary complex undergoes a rate-limiting conformational change followed by a succession of rapid steps in which primer terminus elongation is completed without the participation of nucleotidyl-enzyme covalent attachment.

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Thymine Lesions Produced by Ionizing Radiation in Double-Stranded DNA

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ABSTRACT: A DNA glycosylase which catalyzes the release of thymine residues damaged by ring saturation, fragmentation, or ring contraction from double-stranded DNA has been used to characterize such base derivatives in γ-irradiated DNA. It is shown by chromatographic analysis that irradiation of DNA in neutral solution generates the ring-saturated forms *cis*-thymine glycol, *trans*-thymine glycol, and a monohydroxydihydrothymine, probably 6-hydroxy-5,6-dihydrothymine. The latter compound is only observed after irradiation under hypoxic conditions. The ring-contracted thymine derivative 5-hydroxy-5-methylhydantoin is also formed, and it is the major lesion after irradiation of DNA under O_2. Ring-fragmented products such as methyltartronylurea were only generated in small quantities. Isolation and analysis of the DNA from γ-irradiated human cells also revealed the formation of ring-saturated thymine derivatives, but 5-hydroxy-5-methylhydantoin was not found in this case.

An important component of cellular injury following ionizing radiation consists of base damage in DNA (Cerutti, 1976). This type of radiation damage has not been characterized as extensively as DNA strand breaks or large-scale chromosomal alterations, at least in part because of the technical difficulties involved in the detection and analysis of the lesions. Studies with prokaryotes and lower eukaryotes which permit detailed genetic analysis have indicated that base substitutions are the most common type of mutational event caused by ionizing radiation; both transitions and transversions occur, at A·T as well as at G·C base pairs (Glickman et al., 1980). In general, A·T pairs appear more susceptible than G·C pairs, as judged from the ability of various tester strains employed in the "Ames test" to detect mutations caused by oxidative damage and ionizing radiation (Levin et al., 1982). DNA pyrimidine residues seem more sensitive to radiation than purines, and major pathways of their degradation involve the saturation of the 5,6 double bond and fragmentation of the pyrimidine ring (Scholes, 1976; Teoule et al., 1977).

Quantitative assessments of the spectrum of base lesions produced in DNA by ionizing radiation face the problem that several products are acid labile and decompose into secondary derivatives during chemical hydrolysis. To circumvent this difficulty, we have used a reagent enzyme of broad specificity which releasese altered thymine residues in free form from double-stranded γ-irradiated DNA under mild conditions. The released material has been characterized by chromatographic analysis. The enzyme employed is *Escherichia coli* endonuclease III (also called thymine glycol–DNA glycosylase, urea–DNA glycosylase, and X-ray endonuclease), which is the product of the *nth*$^+$ gene (Cunningham & Weiss, 1985). This enzyme is specific for double-stranded DNA and catalyzes the release of thymine residues damaged by ring saturation, fragmentation, or ring contraction by cleavage of the appropriate glycosyl bonds; in addition, the enzyme has an associated endonuclease activity for apyrimidinic and apurinic sites (Demple & Linn, 1980; Breimer & Lindahl, 1980, 1984; Katcher & Wallace, 1983).

Experimental Procedures

Materials and Reference Compounds. [*methyl*-^{3}H]TTP (45 Ci·mmol^{-1}), [2-^{14}C]TTP (50 mCi·mmol^{-1}), [*methyl*-^{3}H]thymidine (90 Ci·mmol^{-1}), and [*methyl*-^{14}C]thymine (58 mCi·mmol^{-1}) were obtained from Amersham. *E. coli* DNA polymerase I, pancreatic DNase I, and S1 nuclease were purchased from Boehringer. *E. coli* endonuclease III was purified as described (Breimer & Lindahl, 1984). *cis*-Thymine glycol was prepared from [*methyl*-^{14}C]thymine by OsO_4 treatment (Burton & Riley, 1966). To generate the trans form, this compound was isomerized by heating in aqueous solution at 95 °C for 4 h (Barszcz et al., 1963). 6-Hydroxy-5,6-dihydrothymine was observed (by chromatography) as one of the major products of [^{14}C]thymine γ-irradiated under N_2, in agreement with the results of Nofre & Cier (1966), and this compound was purified by reverse-phase high-pressure liquid chromatography (HPLC) on a Micro-Pac MCH-10 column with water as eluant. 5-Hydroxy-5-methylhydantoin was obtained by condensation of [^{14}C]urea with pyruvic acid

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