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Elementary Steps in the DNA Polymerase I Reaction Pathway†

F. R. Bryant, K. A. Johnson, and S. J. Benkovic*

ABSTRACT: The polymerization reaction catalyzed by *Escherichia coli* DNA polymerase I (Pol I) has been studied by using the homopolymer template-primer system poly(dA)·oligo(dT). Isotope-partitioning experiments indicate that the reaction follows an ordered mechanism in which Pol I first combines with template-primer to form an E·poly complex followed by addition of MgTTP and catalysis. The parameters governing the binding of Pol I to the template-primer are k_{on} = 1.2 × 10^6 M^{-1} s^{-1}, k_{off} = 0.25 s^{-1}, and K_D = 2 × 10^{-7} M. Efforts to demonstrate the catalytic competence of the binary E·MgTTP complex were unsuccessful. Following initiation of the catalytic cycle, Pol I catalyzes the incorporation of an average of 40–50 TTP molecules into polymer before dissociating from the template-primer. The processive nature of the polymerization reaction as reflected by the isotope-trapping time dependence can be accounted for by a model in which processive synthesis is treated as a simple partitioning between continued polymerization (k_{cat} = 3.8 s^{-1}, 22 °C) and dissociation of the enzyme from the template-primer under steady-state conditions (k_{off}^{ss} = 0.1 s^{-1}). The rapid quench time course of the polymerization reaction (k_{cat} = 2.5 s^{-1}, 20 °C) exhibited a pre-steady-state burst consistent with two partially rate-determining steps, one of which precedes the actual chemical phosphodiester bond-forming step (k = 4.6 s^{-1}) and the other which follows this step (k = 4.0 s^{-1}). Binding of MgTTP to the E·poly complex was shown to be a rapid equilibrium step by steady-state isotope-partitioning experiments. This suggested that the first rate-determining step may be a first-order isomerization which follows the binding of substrates and precedes bond formation.

Escherichia coli DNA polymerase I (Pol I),[1] an enzyme required for both the repair and the replication of DNA in vivo, has been the most extensively studied of all DNA polymerases. Pol I catalyzes the addition of mononucleotide units derived from deoxyribonucleotide 5′-triphosphates to the 3′-hydroxyl terminus of a primer chain. This reaction requires a template chain which directs the enzyme in its selection of the specific triphosphates according to the Watson–Crick base-pairing rules. In addition to the polymerization activity, Pol I also possesses 3′ → 5′ and 5′ → 3′ exonuclease activities, all residing on a single 109 000-dalton polypeptide chain (Kornberg, 1980).

The mechanism of Pol I has been investigated by a number of approaches including product size measurements (Das & Fujimura, 1979; McClure & Chow, 1980), stereochemistry (Burgers & Eckstein, 1980), and kinetic analyses (McClure & Jovin, 1975; Travaglini et al., 1975; Bambara et al., 1976, 1978). Steady-state kinetic analyses of the Pol I reaction are complicated not only by the multiple catalytic activities of the enzyme but also by the processive nature of the reaction in which Pol I apparently translocates along the template while incorporating many nucleotide residues before dissociation. In the present study, we have carried out isotope-trapping (Rose, 1980; Wilkinson & Rose, 1979) and rapid-kinetic experiments in an effort to identify the elementary steps involved in the initiation and propagation of a processive cycle of polymerization as catalyzed by Pol I. In order to simplify this initial investigation, the homopolymer template-primer system poly(dA)·oligo(dT), which requires only a single triphosphate (TTP) as the reacting nucleotide substrate, was employed.

Experimental Procedures

Materials

DNA polymerase I was purified from *E. coli* 594 (λ *Pol* A *c* 1857 *nin* 5 *Q* am 73 *5* an *7*) according to a published procedure (Davis et al., 1980). The *E. coli* strain was generously furnished by Professor R. Davis, Stanford. The enzyme was judged to be ~95% pure by polyacrylamide gel electrophoresis. Protein concentrations were determined from

† From the Department of Chemistry and the Department of Biochemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received December 22, 1982.* This research was supported by Grants GM-13306 (S.J.B.) and GM-26726 (K.A.J.).

* Address correspondence to this author at the Department of Chemistry.

[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; E·poly, binary Pol I·template-primer complex; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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A_{280} measurements using the extinction coefficient of A_{280} = 0.85 for a 1 mg/mL solution as previously reported (Jovin et al., 1969a,b).

$(dA)_{1000}$, $(dT)_{10}$, and poly(dAT) were from P-L Biochemicals. [^{3}H]TTP and [^{3}H]dATP were from ICN Radiochemicals. TTP and calf thymus DNA were from Sigma. DE81 filter papers were from Whatman. TTPαS (S_P and R_P) and $(dT)_{10}$[^{3}H]dT were furnished by Amar Gupta and Bob Henrie of this laboratory.

Methods

Polymerization Assays. The components of the polymerization reaction solutions are described below; specific experiments are described in the figure legends. All reactions were carried out at 22 °C unless otherwise indicated. Reactions were generally followed by withdrawing aliquots at desired time intervals and quenching by the addition of EDTA to a final concentration of 25 mM. The quenched reaction solutions were then assayed for the incorporation of [^{3}H]TTP into a polymeric form by the method of Brutlag & Kornberg (1972). The quenched solutions were applied to 2.5-cm circles of Whatman DE81 paper. These papers were then washed 3 times by gentle agitation for 5 min in 0.3 M ammonium formate (pH 8.0). This procedure removes unpolymerized [^{3}H]TTP from the papers while leaving polymer bound. 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 by liquid scintillation counting in 5 mL of a standard toluene scintillation fluid.

When parallel polymerization reactions were run and assayed by using either the DE81 paper assay or the more standard acid precipitation assay (Jovin et al., 1969a), identical results were obtained. In a control experiment, a sample of the labeled primer-template system, poly(dA)·$(dT)_{10}$[^{3}H]dT, was recovered in only 5% yield when carried through the acid precipitation assay even when carrier DNA was employed. The same template-primer system was recovered in 100% yield when carried through the DE81 paper assay. When a sample of the $(dT)_{10}$[^{3}H]dT primer alone was carried through the DE81 assay, only ~30% recovery was obtained, indicating that the $(dT)_{10}$[^{3}H]dT primer must be complexed with the high molecular weight poly(dA) template in order to be quantitatively retained on the filters. Since the experiments described in this study required analysis of $(dT)_{10}$ primers elongated by a limited number of nucleotides, the DE81 paper assay was employed throughout.

The components of the polymerization reaction solutions are given below.

Buffer. All reactions were carried out in Tris-HCl (pH 7.4) at a concentration of 50 mM.

$MgCl_2$. The Mg concentration used was 2 mM, in order to give optimal rates of polymerization as previously reported (Travaglini et al., 1975). When high concentrations of TTP were used, the total Mg concentrations was adjusted for the formation of the Mg–TTP complex, so that the free Mg concentration was always 2 mM.

TTP. The TTP concentration was 33 μM unless indicated otherwise. This concentration was found to give optimal rates of polymerization when the poly(dA)·oligo(dT) template-primer system was used. TTP concentrations higher than 33 μM resulted in substrate inhibition.

Template-Primer. The $(dA)_{1000}$·$(dT)_{10}$ template-primer system was used throughout this study. The initial rate of polymerization was found to vary by less than 2-fold when the concentration of 3′-primer termini was kept constant and the ratio of primer to template was increased from 1 to 100, indicating that nonproductive binding of Pol I to single-strand poly(dA) stretches, if present, was not kinetically significant. The ratio of primer to template molecules was 1 unless indicated otherwise. All template-primer concentrations are expressed in terms of 3′-hydroxyl primer termini.

Exonuclease Control Assay. The following exonuclease controls were performed. In one experiment, the template-primer poly(dA)·$(dT)_{10}$[^{3}H]dT, was incubated with Pol I in 50 mM Tris-HCl (pH 7.4). At intervals ranging up to 15 min, 50-μL aliquots were withdrawn and quenched by addition to 12 μL of EDTA (0.1 M). The quenched solutions were applied to DE81 papers and analyzed for radiolabel remaining in a polymeric form. There was no detectable loss of label over this time period, showing that the primer was not subject to exonuclease degradation during the preincubation period. In a second experiment, poly(dA)·$(dT)_{10}$[^{3}H]dT, was preincubated with Pol I in the presence of either 2 mM $MgCl_2$ or 2 mM $MgCl_2$ and 33 μM unlabeled TTP. In both cases, 50-μL aliquots were removed, quenched with 12 μL of EDTA (0.1 M), and assayed for radiolabel remaining in polymeric form. In both cases there was no detectable loss of label, indicating that hydrolysis of the 3′-hydroxyl primer terminus does not occur during the initiation and propagation of the polymerization reaction in this system.

E·TTP-Trapping Experiments. A solution containing 200 nM Pol I and 500 μM TTP (1000 cpm/pmol) was preincubated for 10 min at 22 °C (step 1). Then 10-μL aliquots were withdrawn and added to polyethylene microtubes. To each sample, 90-μL aliquots of a solution containing 222 nM poly(dA)·oligo(dT) and 500 μM unlabeled TTP were added to give 100 μL of reaction solution containing 20 nM Pol I, 200 nM poly(dA)·oligo(dT), and 500 μM TTP (100 cpm/pmol) (step 2). After time intervals of 5, 10, or 15 s the reaction solutions were quenched by addition to 25 μL of EDTA. The reaction solutions were then spotted on DE81 papers and assayed for [^{3}H]TTP incorporation. The control experiment was performed in the same way except unlabeled TTP was used in step 1 and [^{3}H]TTP (111 cpm/pmol) was used in step 2. This resulted in a final reaction solution having TTP of the same specific activity (100 cpm/pmol) as the first experiment. All solutions contained 50 mM Tris-HCl, pH 7.4, and 2.5 mM $MgCl_2$.

E·Polymer-Trapping Experiments. A solution containing 34 nM DNA polymerase and 270 nM poly(dA)·oligo(dT) was preincubated at 22 °C. The results were found to be independent of the preincubation period over a range of 30 s to 15 min; a standard preincubation period of 5 min was used. Then 37-μL aliquots were withdrawn and added to polyethylene microtubes. For the normal polymerization reaction, 13-μL aliquots of a second solution containing 125 μM [^{3}H]TTP and 7.5 mM $MgCl_2$ were added to each sample to give 50 μL of reaction solution. For the polymer-trapping reaction, the second solution also contained 250 μg/mL denatured calf thymus DNA. For the background incorporation control, the first solution contained 86 μg/mL heat-denatured calf thymus DNA. The final reaction solutions contained 25 nM DNA polymerase, 200 nM poly(dA)·oligo(dT), 2 mM $MgCl_2$, and 33 μM [^{3}H]TTP (and 65 μg/mL denatured calf thymus DNA when applicable). All reaction solutions were quenched by addition to 12 μL of 0.1 M EDTA after selected time intervals ranging from 5 to 60 s. The quenched solutions were then applied to DE81 papers and analyzed for [^{3}H]TTP incorporation.

The poly(dA)-oligo(dT) concentration dependence study presented in Figure 3 was performed exactly as described above

for the polymer trapping experiment except that the poly(dA)·oligo(dT) concentration was varied from 68 to 817 nM in the preincubation. The "plateau" values are derived from the total [^{3}H]TTP incorporation ($t \geq 30$ s) achieved.

In order to directly measure the final length of the elongated primers formed under polymer-trapping conditions, a reaction identical with the polymer-trapping reaction shown in Figure 2 was carried out. Two 50-μL samples quenched at 45 s were combined, and the elongated $(dT)_{10}$ primers were analyzed according to the procedure of Das & Fujimura (1979).

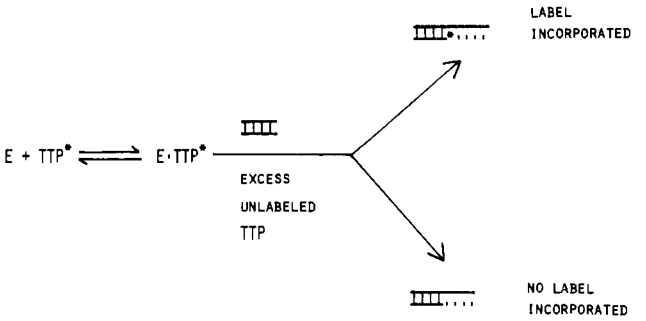
Rapid Quench Experiments. Rapid quench experiments at times >50 ms were performed with a Durrum multimixer apparatus as modified in this laboratory (Benkovic et al., 1974). Runs were performed at ambient temperatures (20 °C) by mixing equal volumes of enzyme–polymer and Mg–[^{3}H]TTP solutions, followed by rapid quenching with 2 volumes of 0.1 M EDTA. Approximately 0.1 mL of quenched solution was collected per time point and assayed by the DE81 paper assay. The stock enzyme–polymer solution contained 400 nM poly(dA)·oligo(dT) and 66 nM DNA polymerase in 50 mM Tris-HCl (pH 7.4). The stock Mg–TTP solution contained 66 μM [^{3}H]TTP (1000–1200 cpm/pmol) and 4 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.4). The final reaction solutions contained 50 mM Tris-HCl (pH 7.4), 33 nM DNA polymerase, 200 nM poly(dA)·oligo(dT), 2 mM $MgCl_2$, and 33 μM [^{3}H]TTP.

Rapid quench experiments at times between 5 and 50 ms were performed by using a three-syringe quench flow apparatus constructed in this laboratory [K. Johnson (unpublished results)] with a design similar to one described by Lynn & Taylor (1970). The progress of the pistons was monitored by using a linear resistor and an oscilloscope and was used to calculate the reaction time for each sample. The temperature was maintained at 22 °C with a circulating water bath. Equal volumes (200 μL) of the enzyme–polymer and Mg–[^{3}H]TTP solutions were mixed to initiate the reaction followed by 400 μL of 0.1 M EDTA to quench. Product was isolated as described above.

Acid quenching, as is normally used in rapid quench experiments, was unsuitable for the present study for the reasons described above. The following control experiment demonstrated that for the present study, EDTA quenching gives results identical with those obtained by acid quenching. Rapid quench reactions were set up exactly as described above and were quenched at $t = 600$ ms in one of two ways. One set (five replicates) was quenched with 0.1 M EDTA, and the other set was quenched with 5% perchloric acid. The quenched samples (80 μL each) were collected, and to each was added an equivalent volume (40 μL) of the alternate quenching solution. All samples were then neutralized with NaOH, applied to DE81 papers, and analyzed for the incorporation of [^{3}H]TTP into polymer. The analysis of the two sets of points differed by less than 5%, even though the time scale of the reaction was only long enough to allow < two enzyme turnovers. This control experiment clearly demonstrates that EDTA is as efficient a quenching agent as perchloric acid for this system. The recovered counts for the individual time points were approximately one-third those recovered under conditions where EDTA quenching was used without subsequent acid treatment, illustrating the problems of quantitation that is encountered due to partial precipitation of the short oligomers.

The TTP chase experiment was performed by mixing equal volumes of an enzyme–polymer solution and a Mg–TTP solution exactly as described above. After 600 ms the reaction solution was mixed with 2 volumes of chase solution containing 297 μM unlabeled TTP and 2.3 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.4). After time intervals ranging from 3 to 10 s, the solutions (100 μL) were quenched manually by addition of 25 μL of 0.1 M EDTA followed immediately by vortexing. The quenched solutions were then assayed for [^{3}H]TTP incorporation. The control points ($t = 0$) were obtained simply by quenching with EDTA at 600 ms, as described above.

Scheme I



Stopped-Flow Light Scattering. Light scattering at 90° to the incident beam was measured in a stopped-flow fluorometer constructed in this laboratory (Porter & Johnson, 1983). Equal volumes (100 μL) of reactants were mixed with a ball-type mixer (Berger et al., 1968) and then flowed to a 2-mm square observation cell. The dead time of the apparatus was 1–2 ms. Light scattering at 295 nm was isolated by using interference filters (10-nm half-bandwidth). The samples and the observation cell were thermostated at 22 °C with a circulating water bath, and the temperature in the sample chamber was measured with a thermocouple. The data were collected with an On-Line Instrument Systems data acquisition system and were fit to a single exponential by the method of moments (K. A. Johnson, unpublished results; Dyson & Isenberg, 1971).

For the association kinetics experiment, one syringe contained 2 μM Pol I and the other contained 4.0 μM oligo(dT) plus 0.08 μM poly(dA) (50:1 primer to template ratio). The base-line light scattering level corresponding to no association ($t = 0$) was obtained by summing the light scattering intensities measured when buffer was substituted for either of the two reactant solutions. All solutions contained 50 mM Tris-HCl (pH 7.4).

Results

E·TTP Trapping. DNA polymerase I was incubated with [^{3}H]TTP in order to form the E·[^{3}H]TTP complex. The enzyme concentration was 200 nM and the TTP concentration was 500 nM, sufficient to give ~86% conversion of the enzyme to the E·[^{3}H]TTP complex according to the dissociation constant of 81 μM previously reported (Englund et al., 1969). The reaction was initiated by diluting this solution 10-fold into a solution containing an equivalent concentration of unlabeled TTP and saturating poly(dA)·oligo(dT) (200 nM). Reactions were allowed to proceed for 5, 10, and 15 s before quenching with EDTA. As shown in Scheme I, upper line, any [^{3}H]TTP which remains bound to the polymerase upon addition of the chase solution will retain the original high specific activity, and if this E·[^{3}H]TTP complex is catalytically competent, it could combine with poly(dA)·oligo(dT), resulting in the incorporation of the [^{3}H]TTP into polymer. If, however, the kinetic ordering of the mechanism requires poly(dA)·oligo(dT) to bind to the polymerase before TTP, then the [^{3}H]TTP must dissociate from the enzyme before any reaction can occur. This would result in the dilution of the [^{3}H]TTP by the unlabeled

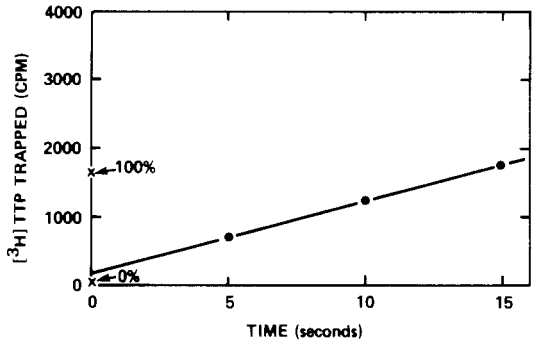


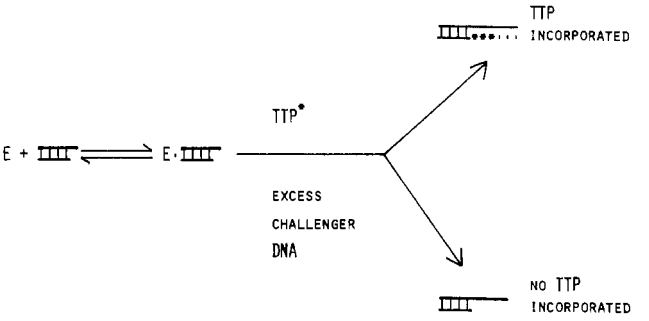
FIGURE 1: E·TTP trapping. The solid line represents the least-squares fit to the TTP-trapping data. 0% represents the extrapolated intercept of the control reaction. 100% represents the expected intercept of the TTP-trapping data if all of the original E·[^{3}H]TTP had continued to product. Experimental details are given in the text.

TTP pool, and no high specific activity [^{3}H]TTP would be incorporated into polymer. The amount of diluted [^{3}H]TTP incorporated into polymer due to normal enzyme turnover was measured in control experiments in which polymerase was preincubated with unlabeled TTP and then diluted 10-fold into a solution containing saturating poly(dA)·oligo(dT) and diluted [^{3}H]TTP having the same final specific activity as in the first experiment.

The results of these experiments are shown in Figure 1. The incorporation data were extrapolated to zero time in order to correct the total [^{3}H]TTP incorporation for the [^{3}H]TTP incorporation due to turnover of the diluted pool. The difference in the extrapolated intercepts of the trapping reaction and the control experiments represents the incorporation due to the reaction of the initial E·[^{3}H]TTP complex. The control reaction, as expected, gave a near zero intercept of only 35 cpm. The trapping reaction gave an intercept of 187 cpm, a difference of 152 cpm. The expected difference, if all of the preformed E·[^{3}H]TTP reacted to give incorporation, would be 1600 cpm. The results therefore show that within experimental error, there was no significant additional [^{3}H]TTP incorporation in the trapping experiment relative to the background control. This suggests that the E·TTP complex is not capable of reacting with poly(dA)·oligo(dT) to give TTP incorporation but instead the kinetic ordering requires the E·poly complex to form first with TTP combining with this complex to give reaction. An alternative explanation that cannot be rule out is that the E·TTP complex is catalytically reactive but that dissociation of [^{3}H]TTP from either the E·[^{3}H]TTP or E·poly·[^{3}H]TTP complex occurs much faster than phosphodiester bond formation, resulting in equilibration of the [^{3}H]TTP with the nonradioactive pool before incorporation can occur (Rose, 1980).

E·Polymer Trapping. DNA polymerase I was incubated with poly(dA)·oligo(dT) in order to form the E·poly complex. This solution was then added to a second solution containing [^{3}H]TTP and denatured challenger DNA. The challenger DNA binds free enzyme and prevents it from carrying out polymerization, thereby serving as a trapping agent for free enzyme (the denatured DNA cannot support polymerization in the absence of three of the four required NTP's). As shown in Scheme II, upper line, if E·poly complex is catalytically competent, then TTP should bind to the complex and initiate the catalytic cycle, resulting in the incorporation of [^{3}H]TTP into polymer. If, however, the kinetic ordering requires TTP to bind first, then the enzyme must dissociate from the poly(dA)·oligo(dT) before any reaction can occur. If this happens the enzyme will be trapped by the challenger DNA and no [^{3}H]TTP will be incorporated into polymer.

Scheme II



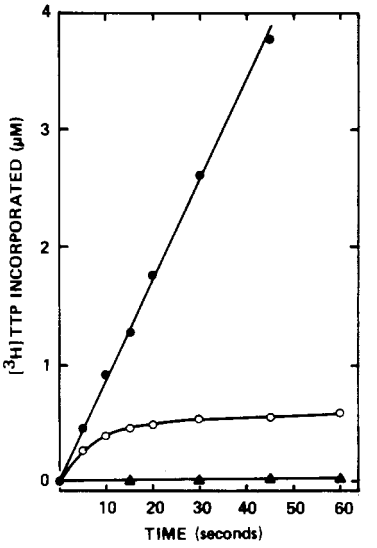


FIGURE 2: E·polymer trapping. Preincubation solutions contained 270 nM poly(dA)·oligo(dT) and 34 nM polymerase. (●) The normal polymerization time course obtained when the reaction is initiated by the addition of MgTTP. (○) The time course obtained when MgTTP and challenger DNA are added simultaneously. (▲) The time course obtained when DNA polymerase is preincubated with poly(dA)·oligo(dT) and challenger DNA before the addition of MgTTP. Final reaction solutions contained 200 nM poly(dA)·oligo(dT), 33 μM [^{3}H]TTP, 2 mM $MgCl_2$, 25 nM DNA polymerase, and 65 μg/mL challenger DNA. Experimental details are given in the text.

The results of this experiment and two controls are shown in Figure 2. When the reaction is initiated with [^{3}H]TTP, but no challenger DNA, the normal uninterrupted polymerization time course is obtained (k_{cat} = 3.8 s^{-1}, 22 °C). When enzyme is added to a mixture of poly(dA)·oligo(dT) and challenger DNA, prior to the addition of [^{3}H]TTP, very little incorporation of [^{3}H]TTP into polymer is found, demonstrating the effectiveness of the challenger DNA trap. When enzyme is preincubated with poly(dA)·oligo(dT) prior to the addition of [^{3}H]TTP and challenger DNA, incorporation of [^{3}H]TTP into polymer is observed to initially proceed normally but then levels off to a plateau value. This behavior may be interpreted as resulting from the enzyme bound to the poly(dA)·oligo(dT) incorporating [^{3}H]TTP into polymer for the duration of one processive cycle, then dissociating from the polymer, and being trapped by the challenger DNA. Note there is no detectable break in the normal polymerization time course corresponding to the time interval of the processive cycle observed in the trapping experiment.

The net incorporation of TTP is dependent on the concentration of E·poly formed in the preincubation and the efficiency of converting TTP to product. This efficiency is dependent on the rate of polymerization, and, hence, the concentration of TTP. At fixed TTP, the net incorporation of TTP will be

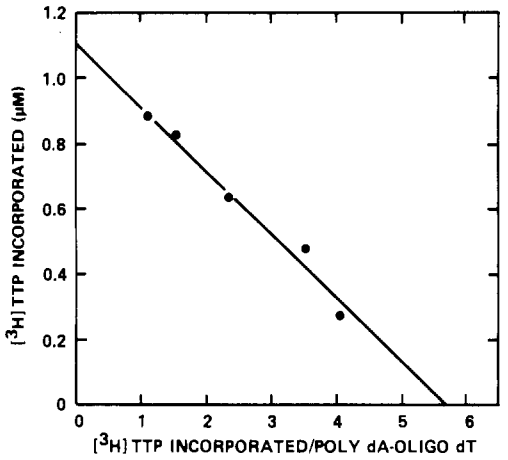


FIGURE 3: Scatchard plot of the final plateau values obtained in the polymer trapping experiment as a function of the initial poly(dA)·oligo(dT) concentration (68–817 nM). The solid line represents the least-squares fit to the experimental data. From the x axis intercept, there are 45 TTP molecules incorporated into polymer per enzyme molecule at saturation with poly(dA)·oligo(dT). The slope gives an intrinsic dissociation constant of 200 nM for the E·polymer complex.

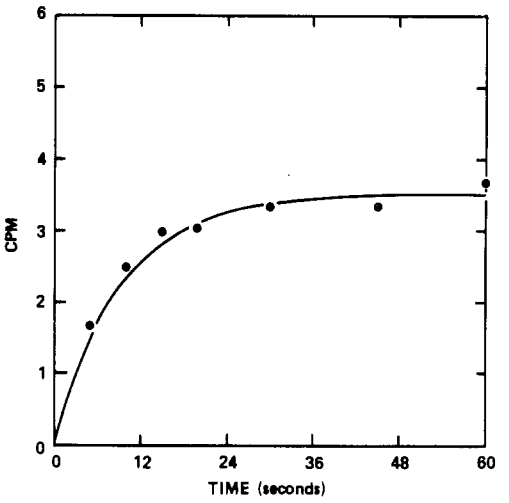


FIGURE 4: Time course of polymer trapping. The curve through the polymer-trapping data (obtained from an experiment identical with that described in Figure 2) represents the theoretical curve calculated from the relationship $N/E = (k_{cat}/k_{off}^{ss})(1 - e^{-k_{off}^{ss}t})$ where N/E = 40 nucleotides per enzyme–polymer complex, k_{cat} = 3.8 s^{-1}, and k_{off} = 0.1 s^{-1} (see Appendix).

proportional to [E·poly] complex. The total amount of [^{3}H]TTP incorporated into polymer, as indicated by the final plateau values, was studied as a function of polymer concentration in the preincubation at a fixed enzyme and TTP concentration. The results of a series of experiments in which the poly(dA)·oligo(dT) concentration was varied from 50 to 600 nM (primer ends) showed saturation with respect to the polymer concentration. The data could be represented by a linear Scatchard plot (Figure 3), indicating that the data could be expressed satisfactorily as a simple bimolecular binding of enzyme to poly(dA)·oligo(dT) to form an E·poly complex. Under the assumption that the total [^{3}H]TTP incorporated into polymer at a given polymer concentration is proportional to the amount of E·poly complex formed in the preincubation, this plot gives an intrinsic dissociation constant K_D = 200 nM for the E·poly complex and an incorporation of 45 TTPs into polymer per enzyme molecule at the saturating poly(dA)·oligo(dT) concentration.

Increasing the [^{3}H]TTP concentration from 33 to 67 or 100 μM resulted in no increase in the total amount of TTP incorporated in polymer, demonstrating that the data obtained at 33 μM TTP were already saturating with respect to converting E·poly to product; that is, all of the E·poly present enters the catalytic cycle upon addition of TTP, rather than dissociates with trapping of the resulting free enzyme by the challenger DNA.

The length of the elongated product was also measured directly by modification of the method of Das & Fujimura (1979). The trapping reaction was carried out, and when the plateau was reached, the reaction was quenched and the poly(dA)·oligo(dT) was isolated by oligo(dT)–cellulose chromatography. The length of the elongated primer was determined by sequential digestion by micrococcal nuclease and spleen phosphodiesterase. The length of elongation is given by the ratio of ([^{3}H]TMP + [^{3}H]Thy)/[^{3}H]Thy. Since the polymerization was done in the presence of challenger DNA, any primer that was elongated was only elongated for one processive cycle. The result of this analysis gave an average elongation length of 50 (±5) nucleotides and represents the processivity of the polymerase under these conditions.

The close agreement between the direct measurement of the elongated product length (50) and the number of TTPs incorporated per enzyme molecule at saturating levels of poly(dA)·oligo(dT) (45) suggests that at saturating levels of poly(dA)·oligo(dT), at least 90% of the enzyme molecules are productively complexed and undergo catalysis upon addition of TTP. No nonproductive binding of the polymerase to the poly(dA)·oligo(dT) template-primer system was apparent. The processivity, as measured by the polymer-trapping experiment, was found to vary between 40 and 50 in various experiments.

A simple model for the processivity of DNA polymerase I is that the processive behavior is the result of a competition between the rate of incorporation of another nucleotide into polymer and the rate of dissociation of the enzyme–polymer complex. If this simple model is correct, the time course of the trapping reaction should follow the relationship

$$N/E = (k_{cat}/k_{off}^{ss})(1 - e^{-k_{off}^{ss}t})$$

where N/E represents the number of nucleotides incorporated per enzyme–polymer complex, k_{cat} is the steady-state rate constant for the polymerization reaction, and k_{off}^{ss} is the rate constant for the dissociation of the enzyme–polymer complex in the steady state (see Appendix). This relationship predicts that at long time intervals, the incorporation of TTP will approach a limiting value, $(N/E)_F = k_{cat}/k_{off}^{ss}$; that is, the number of nucleotides incorporated into polymer in a processive cycle will depend on the ratio of the rate constant for polymerization (k_{cat}) to the rate constant for dissociation of the enzyme from the template-primer. From the data shown in Figure 2, k_{cat} is found to be 3.8 s^{-1} (22 °C) from the control reaction involving no challenger DNA trapping, and the limiting value for N/E is found to be 40 nucleotides per enzyme–polymer complex, when the equilibrium dissociation constant for the preformed enzyme–polymer complex described previously is used. From these values, and the relationship $(N/E)_F = k_{cat}/k_{off}^{ss}$, a value of 0.1 s^{-1} may be calculated for the dissociation rate constant for the enzyme–polymer complex, assuming this simple model. A theoretical curve based on these parameters was calculated for the experiment shown in Figure 2. As seen in Figure 4, the calculated curve matches well with the experimental data, suggesting that the simple model for processivity satisfactorily explains the observed time course and the value of N/E (see Appendix).

The value of 0.1 s^{-1} for the dissociation rate constant was determined in the presence of saturating TTP under steady-state conditions and may represent a weighted sum of the

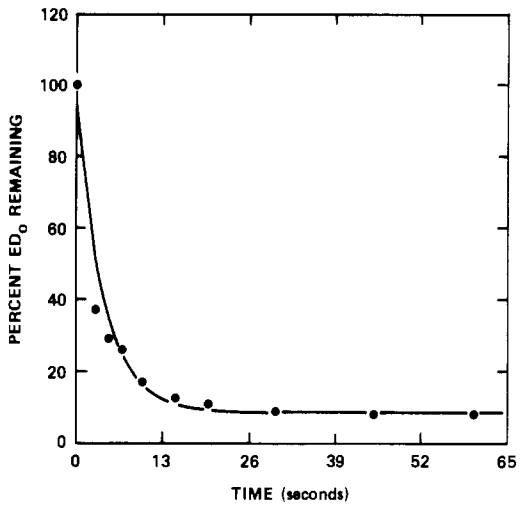


FIGURE 5: Dissociation of the binary E·poly complex as determined by polymer trapping. Experiments were performed as described for the polymer-trapping experiment in Figure 2 except challenger DNA was added first to the preformed E·poly complex and allowed to incubate for varying periods of time before the polymerization reaction was initiated by the addition of MgTTP. The data represent the relative plateau values of TTP incorporation obtained as a function of the time interval between the addition of challenger DNA and MgTTP. The solid line was calculated for a first-order decay process with a rate constant of 0.25 s^{-1}.

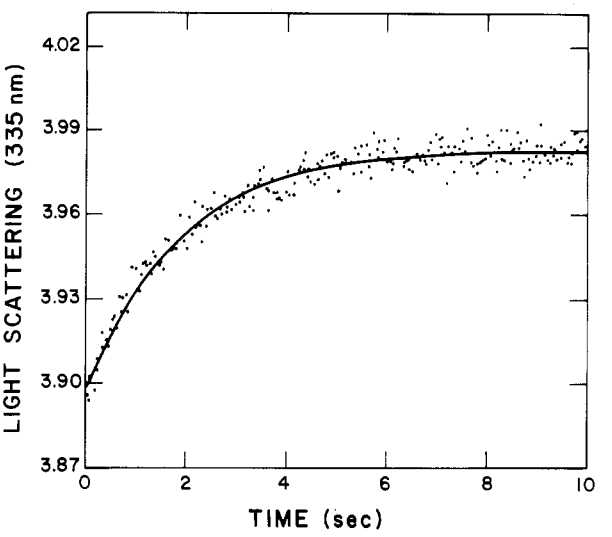


FIGURE 6: Association of Pol I and poly(dA)·oligo(dT) (1:50 molar ratio) as measured by light scattering. The final reaction solution contained 1 μM Pol I and 2 μM template primer (3′ ends) in 50 mM Tris-HCl (pH 7.4). On a time scale of 10 s, 200 data points were acquired. The curve drawn through these points represents a fitting to a single exponential function (k_{obsd} = 0.53 s^{-1}). Experimental details are given in the text.

dissociations of a ternary E·poly·TTP complex and a binary E·poly complex. In order to measure the dissociation rate constant for the initial binary E·poly complex alone, the following variation of the trapping experiment was carried out.

DNA polymerase and poly(dA)·oligo(dT) were preincubated to form the E·poly complex under conditions identical with those in the previous trapping experiments. Then challenger DNA was added to trap the enzyme as it dissociated from the template-primer. At various time intervals, Mg and [^{3}H]TTP were added; any enzyme still complexed with the original poly(dA)·oligo(dT) will undergo the normal processive cycle before dissociating and being trapped, while any enzyme which has already dissociated and been trapped is catalytically silent. After the reaction was allowed sufficient time to reach the final plateau values, the samples were quenched and analyzed for [^{3}H]TTP incorporation. Since the total [^{3}H]TTP incorporation depends on the amount of E·poly still present at the time of addition of Mg and TTP, the amount of total [^{3}H]TTP incorporation as a function of the time interval between addition of challenger DNA and Mg/TTP should represent the decay of the binary E·poly complex. The results of this experiment are shown in Figure 5 and represent a first-order decay of the E·poly complex with a rate constant of 0.25 s^{-1}.

The values of K_D = 200 nM and k_{off} = 0.25 s^{-1} may be used to calculate an association rate constant, k_{on}, of 1.2 × 10^6 M^{-1} s^{-1} for the formation of the enzyme–polymer complex.

Light Scattering Association Kinetics. Macromolecules will scatter incident light, and bimolecular processes involving macromolecular reactants can often be followed in a straightforward way by the observation of a change in the intensity of light scattering. When DNA polymerase I and a 2-fold excess of poly(dA)·oligo(dT) (1:50 molar ratio) were mixed by using a stopped-flow fluorometer in the light scattering mode, a pseudo-first-order increase in light scattering intensity with a rate constant of 0.53 s^{-1} was clearly seen, as shown in Figure 6. By use of this observed first-order rate constant, the previously calculated dissociation rate constant of 0.25 s^{-1}, and the relationship $k_{obsd} = k_{on}[DNA] + k_{off}$, a second-order rate constant of 1.4 × 10^5 M^{-1} s^{-1} can be calculated for the association reaction of DNA polymerase I and this poly(dA)·oligo(dT) template-primer system. Because of the large amount of template-primer required for light scattering measurements, it was not possible to use a 1:1 ratio, as was used in the polymer-trapping experiments for measuring the association rate.

Rapid Quench. The transient kinetics of the polymerization reaction catalyzed by DNA polymerase I were studied in order to examine the incorporation of the first nucleotide of the processive cycle as an event distinct from the further elongation of the primer chain. DNA polymerase and poly(dA)·oligo(dT) were preincubated in order to form the E·polymer complex. This complex was then mixed with Mg and [^{3}H]TTP to initiate the polymerization reaction. The reaction was quenched with EDTA at time intervals ranging from 50 to 1200 ms and then assayed for the incorporation of [^{3}H]TTP into polymer. The conditions are identical with those used to generate the normal polymerization reaction as shown in Figure 2.

From the incorporation curve shown in Figure 7a, there appears to be an initial, faster transient phase which is followed by a slower linear phase. The rate constant of the slower phase (t > 250 ms) is 2.1 s^{-1}, which agrees well with the steady-state rate constant of 2.2 s^{-1} measured manually under identical conditions (20 °C). The early phase was more carefully measured in a second experiment, taking time points from 5 to 50 ms (22 °C). This shorter time resolution allowed the early phase of the reaction to be measured very carefully relative to the steady-state rate. As shown in Figure 7b, the data gave an approximately linear rate which was 1.7 times faster than the steady-state rate. The data also extrapolated to zero TTP incorporation at zero time, verifying that the EDTA quench was working efficiently.

The incorporation data was fit to the simple model

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

where E represents the E·polymer complex, S is MgTTP, and P is the polymer following the incorporation of a nucleotide at the 3′-hydroxyl terminus of the primer. It was assumed that the equilibration of E + S ⇌ ES is sufficiently rapid that this

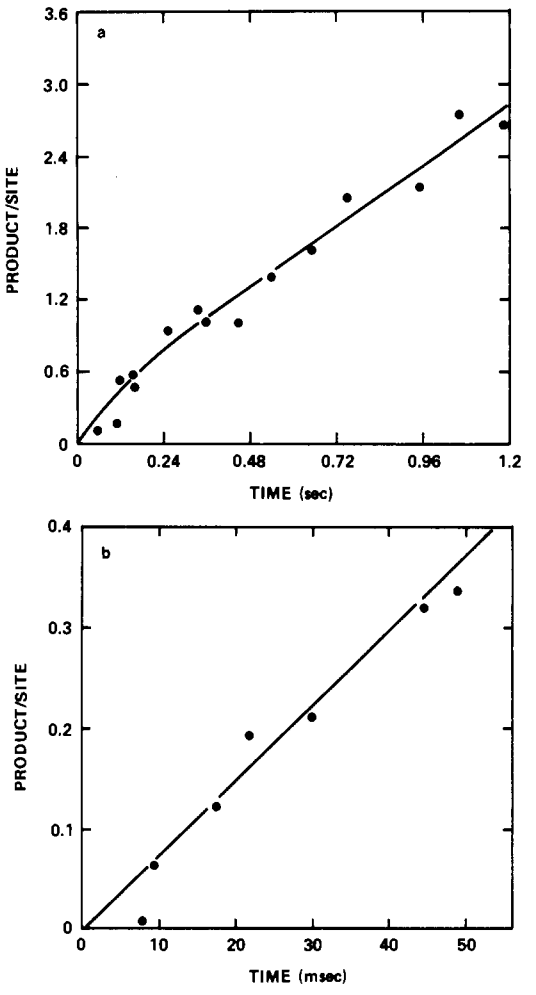


FIGURE 7: (a) Rapid quench time course of net [^{3}H]TTP incorporation into poly(dA)·oligo(dT) catalyzed by DNA polymerase. The final reaction solution contained 50 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 33 μM [^{3}H]TTP, 200 nM poly(dA)·oligo(dT), and 34 nM DNA polymerase (20 °C). The solid line represents a computer fit to the model described in the text, where k_2 = 4.6 s^{-1} and k_3 = 4.0 s^{-1}. Experimental details are given in the text. (b) Early phase of [^{3}H]TTP incorporation catalyzed by DNA polymerase. The reaction conditions were identical with those described in Figure 2 (22 °C). The solid line represents the least-squares fit to the experimental data.

first transient phase can be neglected. Support for this assumption stems from experiments which showed that the rates of neither the initial phase nor the steady-state phase were affected by increasing the concentration of TTP from 25 to 50 μM.

In this model, the first nucleotide of the processive cycle is incorporated at a rate governed by k_2 and by the E $\rightleftharpoons$ ES equilibrium whereas all subsequent nucleotides are incorporated at the steady-state rate, governed by $k_{cat} = k_2k_3/(k_2 + k_3)$. The experimental data can be satisfactorily fit to this model, yielding values for the rate constants of k_2 = 4.6 s^{-1} and k_3 = 4.0 s^{-1}, giving k_{cat} = 2.1 s^{-1} (as shown by the curve drawn in Figure 7a).[2]

[2] The transient incorporation of TTP into polymer as predicted by this model reaction scheme is described by the following rate equation originally derived by Gutfreund (Gutfreund, 1972):

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

In this equation, the steady-state rate constant, k_{cat}, is equal to $k_2k_3/(k_2 + k_3)$, and the extrapolated intercept of the steady-state phase is equal to $[k_2/(k_2 + k_3)]^2[E_T]$. The data were fit by a computer graphics technique (Johnson, 1983).

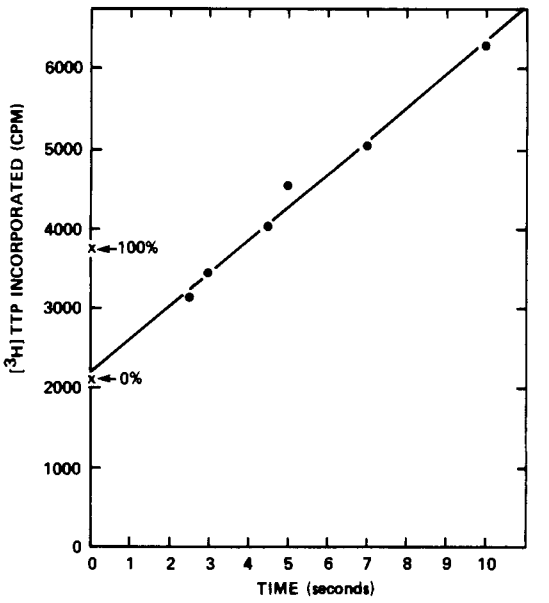


FIGURE 8: TTP chase experiment. The reaction conditions were the same as those described in Figure 5, except that at 600 ms the reaction solutions were diluted with unlabeled TTP (final TTP concentration 165 μM). The reactions were allowed to continue for time intervals ranging from 3 to 10 s before being quenched with EDTA. The solid line represents a least-squares fit to the experimental data. The control point at t = 0 of 2121 cpm was obtained by EDTA quenching at 600 ms. The extrapolated intercept at t = 0 is 2230 cpm. Experimental details are given in the text.

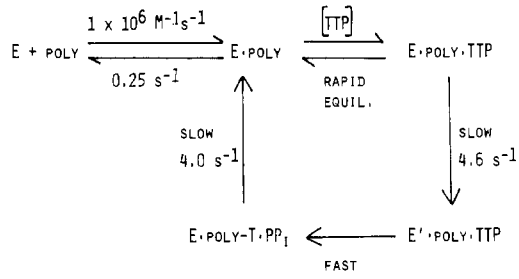
Steady-State TTP Trapping. Polymerization reactions were carried out exactly as described for the previous rapid quench experiments. After a given time interval sufficient to allow the reaction to enter the steady-state phase, as indicated by the previous rapid kinetic time course (t = 600 ms), the reactions were treated in one of two ways. One treatment consisted simply of quenching with EDTA and analyzing for TTP incorporation into polymer. As described previously, this value was shown to be equivalent to that obtained by quenching with perchloric acid and represents the total incorporation of [^{3}H]TTP into polymer up to that time point. The second treatment consisted of the addition of a 5-fold excess of unlabeled TTP after exactly the same time interval. These reactions were allowed to continue for time intervals ranging from 3 to 10 s, before being quenched with EDTA. These data were extrapolated to zero time in order to correct for [^{3}H]TTP incorporation due to turnover of the diluted pool. The difference between the EDTA quench value and the extrapolated intercept of the TTP chase data for a given time point in the steady state represents the amount of enzyme-bound [^{3}H]TTP that is converted to product as opposed to being lost to the unlabeled pool by dissociation.

The data for this experiment are shown in the Figure 8. The point at zero time is the EDTA quench value for the 600-ms time point. The solid line represents a least-squares fit of the TTP chase data at this same time point. The difference between the extrapolated intercept of the TTP chase data (2230 cpm) and the EDTA quench value (2121 cpm) is 109 cpm. The expected difference for one enzyme equivalent of [^{3}H]TTP, based on the enzyme concentration and the specific activity of the [^{3}H]TTP, is 1460 cpm. Thus within experimental error, no additional enzyme-bound [^{3}H]TTP is incorporated into polymer when a TTP chase is employed, rather than quenching the reaction directly.

Discussion

The results of this study are consistent with the minimal reaction scheme for the polymerization reaction catalyzed by

Scheme III



DNA polymerase I shown in Scheme III. The following discussion will present justification for each part of the reaction scheme.

Isotope-trapping experiments clearly demonstrate that Pol I and poly(dA)·oligo(dT) combine to give a catalytically functional E·poly complex in the polymerization reaction. More than 90% of preformed E·poly complex could be trapped by addition of saturating TTP to give an active complex which carried out a processive cycle of polymerization. Pol I is also known to combine with TTP at high concentrations (K_D = 81 μM), to give a binary E·TTP complex (Englund et al., 1969). However, attempts to similarly trap this E·TTP complex by addition of saturating poly(dA)·oligo(dT) were unsuccessful. These results suggest an ordered mechanism for substrate binding in which the template-primer must bind to the polymerase before the nucleoside triphosphate can bind. It may be argued that the E·TTP complex may be catalytically functional but that dissociation of TTP from E·TTP or E·poly·TTP may be very fast relative to catalysis. Nevertheless, a kinetically competent pathway appears to be the ordered addition of template-primer followed by nucleoside triphosphate. This finding is in agreement with conclusions reached by McClure and Jovin on the basis of a steady-state kinetic study of the Pol I reaction using the poly(dAT) template-primer system (McClure & Jovin, 1975). In view of the template-directed nature of DNA polymerase catalyzed synthesis, it is especially reasonable that an ordered mechanism would be followed. Also, in a processive mechanism, after initiation and incorporation of the first nucleotide of the processive cycle, TTP would be required to bind to an E·polymer complex in order to continue processive polymerization. Control experiments showed that no hydrolysis of the 3′-primer terminus of the radiolabeled template-primer, poly(dA)·(dT)$_{10}$[^{3}H]dT, occurs during the initiation and continuation of polymerization catalyzed by Pol I. Also, two groups have reported that TTP is not converted to TMP (does not undergo turnover activity) with this template-primer system during the initial reaction period; the appearance of TMP does not occur until TTP has been depleted (Travaglini et al., 1975; Wang et al., 1974). These results indicate that exonuclease activities play no role in initial catalysis, and therefore no exonuclease hydrolysis steps were included in the reaction scheme.

The processivity of Pol I under the conditions of this study was 40–50 TTPs incorporated per encounter of enzyme with the template-primer, as measured in the polymer-trapping experiments. The time course of the polymer-trapping experiment was consistent with a simple model of processivity where processivity is a function of the ratio of k_{cat} to k_{off}^{ss}. That is, processivity is determined by the affinity of the enzyme for the template-primer relative to the efficiency of continued phosphodiester bond formation. In this analysis, k_{off}^{ss} represents dissociation of the enzyme from the template-primer to the extent that it becomes equilibrated with the components of the reaction solution. Dissociation events in which the enzyme leaves the template-primer but returns due to proximity and domain effects are indistinguishable from the case where the enzyme never leaves the template-primer and therefore do not contribute to the observed value of k_{off}. The measure of processivity obtained from this (and any other) experiment presumably will depend greatly on experimental conditions such as the relative concentration of challenger DNA and statistical likelihood of enzyme dissociating from and returning to the same template-primer in the absence and presence of such challenger DNA (or substrate DNA). These considerations will not affect our determination of K_D for the enzyme–polymer complex since the value of apparent processivity serves only as a proportionality constant for the quantitation of E·poly complex formed at specific concentrations of enzyme and template-primer.

The value of k_{off}^{ss} determined by fitting the polymer-trapping time course to this simple model of processivity was 0.1 s^{-1} and corresponds to dissociation under polymerizing conditions. The value of k_{off} determined for dissociation of the binary E·poly complex under preequilibrium conditions was 0.25 s^{-1}. The equilibrium dissociation constant for the E·poly complex was measured as 2×10^{-7} M from the poly(dA)·oligo(dT) concentration dependence in the polymer-trapping experiment. These values may be used to calculate an association rate constant for formation of the E·poly complex of 1.2×10^6 M^{-1} s^{-1}. This value is in approximate agreement with the association rate constant of 1.4×10^5 M^{-1} s^{-1} measured by stopped-flow light scattering. The 8-fold difference in rate constants may be due to the fact that a higher primer to template ratio was used in the light scattering measurements. These values taken together serve to estimate the equilibrium for the binding between template-primer and enzyme under our conditions.

Our estimates of $K_D = 2 \times 10^{-7}$ M and k_{off} = 0.25 s^{-1} can be compared with the values of $K_D = 1 \times 10^{-8}$ M and k_{off} = 0.004 s^{-1} measured by Das and Fujimura for T5 DNA polymerase (Das & Fujimura, 1980). Also, the T5 enzyme was found to be highly processive (>200). The higher template affinity and processivity of the T5 enzyme may reflect its role as a replication enzyme whereas the lower template affinity and processivity of Pol I may well reflect its role as a repair enzyme responsible for filling in short stretches of gapped DNA.

An examination of the rapid quench time course of the processive cycle that ensues upon addition of TTP to the E·poly complex allowed a breakdown of the steps involved in catalysis. The time course showed a rapid incorporation of the first enzyme equivalent of TTP followed by slower incorporation of the subsequent nucleotides of the processive cycle. This time course is consistent with a minimal mechanism in which there are two partially rate-determining steps of approximately equal magnitude, one of which precedes the phosphodiester bond-forming step and the other which follows this step. The phosphodiester bond-forming step itself was not considered as a candidate for one of the rate-determining steps because of the lack of rate difference between TTP and TTPαS (S_P) under conditions where destabilization of the 3′-hydroxyl primer terminus is not a factor (Burgers & Eckstein, 1980; Bryant & Benkovic, 1983). Thiophosphate esters are known to be less reactive chemically toward nucleophilic displacement reactions than are the analogous phosphate esters because the sulfur ligand is less electronegative than oxygen and thereby renders the phosphorus center less electrophilic and less susceptible to nucleophilic attack (Benkovic & Schray, 1971).

In nearly every phosphoryl transfer enzyme examined with thiophosphate analogues, a reduction in rate has been observed (Knowles, 1980). The lack of a rate difference in the case of Pol I suggests that the chemical step is so fast relative to the rate-limiting steps of the mechanism that a reduction in rate of the chemical step expected by sulfur substitution at the reactive center of the substrate still does not make this chemical step rate limiting.

The bimolecular binding of TTP to the enzyme was also ruled out as the first rate-determining step in several ways. First, the rate of the initial phase of TTP incorporation was not increased by increasing the TTP concentrations. If the first rate-determining step were bimolecular binding, it should show a TTP dependence, k[TTP]. Second, isotope-trapping experiments carried out during the early steady-state phase (first processive cycle) of polymerization at saturating TTP concentrations did not result in enzyme-bound TTP being "chased" into product. This shows that a given enzyme-bound TTP dissociates much faster than it is converted to product, meaning that TTP is in rapid equilibrium with the enzyme. These results are consistent with a mechanism in which a slow step follows the initial rapid TTP binding but precedes the chemical step.

These results provide a simple explanation of why EDTA quenching works just as efficiently as acid quenching in this particular enzyme system. Any free MgTTP in solution of course is readily trapped by added EDTA. Any MgTTP bound to the enzyme at the time of addition of EDTA will dissociate much faster than it goes to product and is also readily trapped. Therefore EDTA quenching gives a measure only of the TTP actually incorporated into polymer at the time of addition, as does acid quenching.

These results are consistent with a mechanism in which the first rate-determining step follows TTP binding and precedes the chemical bond-forming step. This step most likely involves a first-order isomerization of the ternary E·poly·TTP complex probably involving a conformational change. This conclusion provides experimental support for an idea implicit in a proofreading mechanism (Kornberg, 1980). The idea is that the specificity of DNA polymerase is based on base pairing between the template and the nucleotide being incorporated. When a correctly base paired NTP is in the active site, the enzyme may respond possibly by a change in conformation, so that subsequent catalytic steps can occur. If a nonmatching NTP were to bind at the active site, a correct base pair could not form, there would be no conformational change, and the triphosphate would be rejected. The first rate-determining step in our proposed mechanism may correspond to this predicted conformational change.

It is more difficult to assign the second rate-determining step to a specific physical event. One possibility is that it corresponds to translocation of the newly elongated primer out of the TTP binding site, possibly by a conformational change, in order to return the E·poly complex to the original configuration having an open binding site for the next TTP to be incorporated. This step conceivably could be coupled to pyrophosphate release. Another possibility is that the delay after the phosphodiester bond-forming step may be a proofreading period in which misincorporated nucleotides may be excised before the enzymes return to a configuration in which the next nucleotide is incorporated (Brutlag & Kornberg, 1972). Further rapid quench experiments aimed at resolving these possibilities are in progress.

The minimal mechanism for polymerization proposed here may not be appropriate for Pol I under all conditions. For instance, the results of Bambara et al. on the processive polymerization of the cohesive ends of λ DNA reveal a very different mechanistic picture: rapid binding of enzyme to primer template, a long delay before initiation of polymerization because of a shift in equilibrium from inactive to active enzyme, and a cycle of processive polymerization steps before a slow dissociation takes place (Bambara et al., 1976). Nevertheless, our studies here using the simple homopolymer template-primer system provide a useful first approximation of the individual steps that comprise the catalytic cycle.

Appendix

Time Dependence of the Polymer-Trapping Experiment. Processive polymerization is described as a simple competition between continued incorporation of nucleotides into polymer by the enzyme and dissociation of the enzyme from the template-primer under steady-state conditions. This model is illustrated as

$$\begin{array}{ccc} ED_N & \xrightarrow{k_{cat}} & ED_{N+1} \\ \downarrow{\scriptstyle k_{off}^{ss}} & & \\ E & & \end{array}$$

where ED = Michaelis complex, E = enzyme (trapped by challenger DNA), ED_0 = initial concentration of Michaelis complex and = E_T under saturating conditions, and N = nucleotides incorporated into polymer. The dissociation reaction is an irreversible reaction under polymer-trapping conditions.

The rate of incorporation of nucleotide into polymer is given by

$$\frac{dN}{dt} = k_{cat}[ED]$$

The concentration of ED is given by

$$[ED] = [ED]_0 e^{-k_{off}^{ss} t}$$

Substitution gives

$$\frac{dN}{dt} = k_{cat}[ED]_0 e^{-k_{off}^{ss} t}$$

This expression may be integrated to give

$$\int_0^n dN = \int_0^t k_{cat}[ED]_0 e^{-k_{off}^{ss} t}\, dt$$

$$N(t) = \frac{k_{cat}}{k_{off}^{ss}}[ED]_0(1 - e^{-k_{off}^{ss} t})$$

or

$$\frac{N(t)}{[ED]_0} = \frac{k_{cat}}{k_{off}^{ss}}(1 - e^{-k_{off}^{ss} t})$$

where $N(t)/[ED]_0$ = the number of nucleotides incorporated in polymer per initial ED complex as a function of time.

Polymers of length, L_i, are obtained from molecules of ED complex that dissociate at time, t_i, where $L_i = k_{cat} t_i$. The number of complexes that dissociate in an interval of time, $t_2 - t_1$, gives the number of newly synthesized DNA molecules, M, in the range of lengths, $L_2 - L_1$, i.e.

$$M_{L_2 - L_1} = [ED]_{t_2} - [ED]_{t_1}$$

where $[ED]_{t_i}$ is the concentration of Pol I·poly complexes remaining at time t_i so that M follows the first-order decay defined above:

$$M_{L_2-L_1} = [ED]_0(e^{-k_{off}^{ss}t_2} - e^{-k_{off}^{ss}t_1})$$

Introducing the expression for polymer lengths results in the following equation relating M to the length distribution:

$$M_{L_2-L_1} = ED_0(e^{-k_{off}^{ss}L_2/k_{cat}} - e^{-k_{off}^{ss}L_1/k_{cat}})$$

The mean length predicted is $\bar{M}$ = 40 nucleotides for k_{cat} = 4 s^{-1} and k_{off}^{ss} = 0.1 s^{-1}.

Registry No. Pol I, 9012-90-2; MgTTP, 72781-90-9.

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Synthesis of a Cleavable Dinucleotide Photoaffinity Probe of Ribonucleic Acid Polymerase: Application to Trinucleotide Labeling of an *Escherichia coli* Transcription Complex†

Michelle M. Hanna‡ and Claude F. Meares*

ABSTRACT: The cleavable dinucleotide photoaffinity probe 5′-[[(4-azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)uridine was prepared and used to determine the 5′ contacts of a trinucleotide in an *Escherichia coli* RNA polymerase/T7 DNA transcription complex. The probe was prepared by alkylating 5′-(thiophosphoryl)adenylyl(3′–5′)uridine with azidophenacyl bromide. The 5′-(thiophosphoryl)adenylyl(3′–5′)uridine was prepared by the abortive initiation reaction of RNA polymerase on a poly[d(A-T)] DNA template, using adenosine 5′-*O*-(thiomonophosphate) and uridine triphosphate as substrates. A transcription complex containing a radiolabeled trinucleotide at the A1 promoter of bacteriophage T7 D111 or D123 DNA was prepared by using the dinucleotide photoaffinity probe as initiator and cytidine [α-^{32}P]triphosphate as the other substrate. After photolysis, the labeled subunits and DNA were isolated, and the trinucleotide was removed in the presence of phenylmercuric acetate and analyzed by polyacrylamide gel electrophoresis. The 5′ end of the trinucleotide was found to label the DNA (≈88%) and also the β (≈10%) and σ (≈3%) subunits of *E. coli* RNA polymerase. It was also shown that the order of migration of the β and β' subunits of *E. coli* RNA polymerase on polyacrylamide gel electrophoresis in sodium dodecyl sulfate is different from that in sodium dodecyl sulfate plus urea.

RNA polymerase is an oligomeric enzyme which catalyzes the synthesis of RNA, using DNA as a template. *Escherichia coli* RNA polymerase holoenzyme contains five major subunits with a total molecular weight of approximately 454 000 and consists of subunits β' (M_r 160 000), β (M_r 151 000), σ (M_r 70 000), and (two) α (M_r 36 000) (Chamberlin, 1982). The σ subunit is required for specific initiation of transcription at promoter sites on DNA and dissociates shortly thereafter to leave the core enzyme (Travers & Burgess, 1969; Hansen & McClure, 1980). Less is known about the functions of the other enzyme subunits.

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‡ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

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