Kinetic Mechanism of the 3′ → 5′ Proofreading Exonuclease of DNA Polymerase III. Analysis by Steady State and Pre-Steady State Methods†

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ABSTRACT: DNA polymerase III holoenzyme is the major replicative enzyme in *Escherichia coli*. An important component of the high-fidelity DNA synthesis that is characteristic of DNA polymerase III holoenzyme is the 3′ → 5′ proofreading exonuclease activity resident in the ϵ subunit. Steady state and pre-steady state conditions have been used to determine equilibrium and Michaelis constants for substrate binding and the rate constant for cleavage by purified ϵ subunit. The steady state kinetic constants are $K_m = 16 \pm 6$ μM and $k_{cat} = 210 \pm 23$ s^{-1} for degradation of single-stranded DNA by ϵ. These steady state values are in agreement with the rate constants determined for excision of the 3′ nucleotide of a dT_{10} oligomer under pre-steady state conditions. Using a simple two-step model, $E + D_n \rightleftharpoons E{\cdot}D_n \rightarrow E + D_{n-1}$, we find $K = 12$ μM and $k_f = 280$ s^{-1} for the dT_{10} substrate. In these experiments, ϵ subunit acts in a distributive manner and product release is not the rate-limiting step. Activity of the ϵ subunit on paired DNA oligonucleotides with zero to three mismatches at the 3′ terminus indicates that an additional step is required in the mechanism. In the scheme $D_n \rightleftharpoons D_n^* + E \rightleftharpoons E{\cdot}D_n^* \rightarrow E + D_{n-1}$, the 3′ terminus undergoes a conformational change or "melts" before the DNA is a substrate for ϵ subunit. With this additional step, the values for binding of activated substrate and cleavage are the same as those for single-stranded DNA. The kinetics for exonucleolytic degradation of single-stranded, paired, and mispaired oligonucleotides support the model that the rate-limiting step in exonucleolytic proofreading of DNA by ϵ subunit is the DNA-melting step.

DNA polymerase III (pol III)[1] is the replicative enzyme in *Escherichia coli* (Kornberg & Baker, 1992). This enzyme complex is composed of ten distinct proteins arranged in an asymmetric dimer and functions to replicate DNA in a rapid and accurate manner (Maki & Kornberg, 1988; Studwell-Vaughan & O'Donnell, 1991; Fradkin & Kornberg, 1992; Wu et al., 1992). The minimal active complex prepared from the pol III holoenzyme, the catalytic core, is composed of three subunits: α, ϵ, and θ (McHenry & Crow, 1979). The ϵ subunit (*dnaQ* gene product) of pol III contains the 3′ → 5′ proofreading exonuclease activity (Scheuermann et al., 1983; Scheuermann & Echols, 1984). The α subunit catalyzes the 5′ → 3′ DNA-polymerizing activity (Maki & Kornberg, 1985), and the θ subunit has no known enzymatic activity (Studwell-Vaughan & O'Donnell, 1993). The pol III core is capable of accurate DNA synthesis, but this subassembly lacks the high processivity of the pol III holoenzyme (Fay et al., 1981). The additional accessory subunits convert the core to a replicative holoenzyme capable of rapid initiation and highly processive DNA synthesis (O'Donnell, 1992; Burgers & Kornberg, 1982).

The replicating enzyme must duplicate genomic DNA with high accuracy. Replication fidelity by pol III is ensured through correct dNTP selection by the α subunit polymerase and exonucleolytic removal of incorrectly paired nucleotides by the ϵ subunit proofreading exonuclease. Proofreading in pol III contributes about 10^2-10^3-fold to replication fidelity on the basis of *in vivo* and *in vitro* studies using mutated ϵ subunit (Echols & Goodman, 1991). The ϵ subunit, as an isolated polypeptide or as a component of the pol III core or holoenzyme complex, excises the 3′ terminus of single-stranded DNA at a faster rate than it excises that of paired DNA (Scheuermann & Echols, 1984; Maki & Kornberg, 1987). The exonuclease activity of the ϵ subunit on paired or mispaired 3′ termini shows a greater dependence on temperature than the activity on single-stranded DNA (Brenowitz et al., 1991). These observations support the hypothesis that proofreading specificity results from the melting capacity of the 3′ terminus, which is higher for mispaired than paired DNA (Brutlag & Kornberg, 1972; Bessman & Reha-Krantz, 1977).

The ϵ subunit of pol III is the best example of a 3′ → 5′ proofreading exonuclease that physically associates and functionally cooperates with a DNA polymerase activity located on a separate polypeptide. This distinction provides the unique opportunity to study an important step in DNA replication fidelity, exonucleolytic proofreading, in the absence and in the presence of DNA polymerase activity. In this paper, we describe the minimal kinetic scheme for ϵ subunit action on single-stranded DNA oligonucleotides and on paired and mispaired oligonucleotides. These studies

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[1] Abbreviations: pol III, DNA polymerase III; ATP, adenosine triphosphate; TMP, thymidine monophosphate; dNTP, deoxynucleoside triphosphate; dNMP, deoxynucleoside monophosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Table 1: Oligonucleotide Substrates Used for Kinetic Studies of ϵ

single-stranded substrates		
dT_{10}		5′ TTTTTTTTTT 3′
dG_{10}		5′ GGGGGGGGGG 3′
paired substrate		
	GG20mer	5′ TAATACGACTCACTATAGGG 3′
	35mer	3′ ATTATGCTGAGTGATATCCCGATTAACCTCGACCGGT
mispaired substrates		
single mispair	GT20mer	5′ AATACGACTCACTATAGGG**T** 3′
		3′ ATTATGCTGAGTGATATCCCGATTAACCTCGACCGGT
double mispair	GTT21mer	5′ TAATACGACTCACTATAGG**TT** 3′
		3′ ATTATGCTGAGTGATATCCCGATTAACCTCGACCGGT
triple mispair	GTTT21mer	5′ TAATACGACTCACTATAG**TTT** 3′
		3′ ATTATGCTGAGTGATATCCCGATTAACCTCGACCGGT

provide further evidence that the rate-limiting step in exonuclease activity is melting of the duplex DNA.

EXPERIMENTAL PROCEDURES

Materials. The [γ-^{32}P]ATP was from Amersham Corp. The T4 polynucleotide kinase was purchased from Promega. The TMP, dithiothreitol, and bovine serum albumin (BSA, radioimmunoassay grade) were from Sigma. The MonoQ 10/10 column, dG_{10}, and dT_{10} were from Pharmacia LKB Biotechnology Inc. Reverse phase cartridges (Sep-Pak C_{18}) were from Waters.

Synthetic oligonucleotides. Synthetic oligonucleotides (Table 1) were synthesized in the Cancer Center of Wake Forest University. Oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase (Perrino & Mekosh, 1992). Radiolabeled oligonucleotides were purified by electrophoresis through a 20% denaturing polyacrylamide gel. The DNA was electroeluted from gel slices using an Elutrap apparatus (Schleicher and Schuell) in TAE [40 mM Tris-acetate (pH 7.5) and 1 mM EDTA] and desalted using a Sep-Pak C_{18} cartridge. Oligonucleotides were quantified by A_{260} using the following molar extinction coefficients: dT_{10}, $\epsilon = 96\,000$; dG_{10}, $\epsilon = 114\,000$; GG20mer, $\epsilon = 216\,600$; GT20mer, $\epsilon = 216\,600$; GTT21mer, $\epsilon = 246\,700$; GTTT21mer, $\epsilon = 245\,000$; and 35mer, $\epsilon = 443\,700$ M^{-1} cm^{-1}. Oligomer concentrations are expressed as 3′ termini. Duplex DNA substrates were prepared by hybridizing 5′-radiolabeled 20mer or 21mer to the complementary 35mer at a 1:1 molar ratio, heating to 100 °C, and cooling slowly (Perrino & Mekosh, 1992). To determine if purification of duplex DNA was necessary, 20mer or 21mer was hybridized to the 35mer at a 1:1.5 molar ratio, and excess 35mer was removed using a nondenaturing 20% polyacrylamide gel. No experimental differences were detected using duplex DNAs prepared by these two methods.

Purification of ϵ Subunit. The ϵ subunit was purified through the guanidine hydrochloride denaturation−renaturation step from the *E. coli* strain MC1000 containing the plasmids pRK248-*cIts-tet* and pNS360-*dnaQ-amp* as described (Scheuerman & Echols, 1984). The renatured enzyme was dialyzed against buffer A [25 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 1 mM EDTA, 20% glycerol, and 50 mM NaCl] containing 0.2 mM phenylmercurysulfonyl chloride and 1 μg/mL leupeptin and pepstatin A and loaded onto a MonoQ 10/10 column equilibrated with buffer A. The MonoQ column was washed with 50 mL of buffer A and developed with a 100 mL linear gradient of 50 to 300 mM NaCl in buffer A. A single peak of protein eluted from the column at 150 mM NaCl. Peak fractions were pooled and dialyzed against buffer A. The resulting protein was homogeneous by SDS−PAGE and was stored in aliquots at −80 °C, retaining full activity for at least 1 year. The protein concentration was determined by Bradford assay using BSA as a standard. The metal free enzyme, dT_{10}, and buffers were prepared by dialysis against 50 mM Tris-HCl (pH 7.5) containing Chelex-100 (Biorad).

Scheme 1

Scheme 2

Steady state experiments. The standard reaction mixture (10 μL) contained 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 50 μg/mL BSA, and [^{32}P]dT_{10} at 6−90 μM. The ϵ subunit was 1−6 nM and was diluted in a solution containing 50 mM Tris-HCl (pH 7.5) and 0.5 mg/mL BSA. Reactions were performed at 25 °C for the times indicated in the figure legends and were quenched by addition of 20 μL cold of 95% ethanol. Samples were dried *in vacuo* and resuspended in 6 μL of 95% formamide dye mixture. Samples were heated in a boiling water bath for 3 min and subjected to electrophoresis on a 20% polyacrylamide denaturing gel. Dried gels were exposed to Kodak XAR film. Quantitation was performed using an AMBIS radioanalytic scanner (San Diego, CA). All data used were in the linear phase of the reaction curves.

Pre-Steady State Experiments. Pre-steady state kinetic experiments were performed using a rapid-quench-flow apparatus (KinTek Instruments, University Park, PA) and the same reaction buffer as the steady state experiments. The enzyme solution in one loop (27 μL) was mixed with the oligonucleotide substrate in the second loop (27 μL), and the reaction was allowed to proceed at 25 °C for time intervals ranging from 3 ms to several seconds. Reactions were quenched in 0.3 M EDTA (final concentration). A portion of the quenched reaction mixture (5 μL) was mixed with an equal volume of 95% formamide dye solution, and the products were analyzed by electrophoresis through a denaturing 15 or 20% polyacrylamide gel.

Data Analysis. The kinetic data were modeled using the HopKinsim1.7.2 kinetic simulation program (Barshop et al., 1983). All curves were fit to the mechanisms and indicated rate constants in Scheme 1 and Scheme 2. Nonlinear regression was performed using the program Kaliedagraph

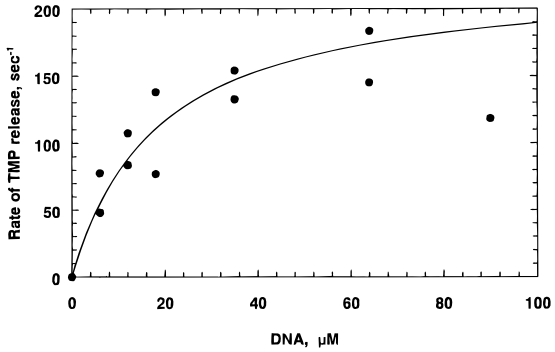

FIGURE 1: Steady state kinetics of dT_{10} degradation by ϵ subunit. Exonuclease reactions containing 1.1 or 2.2 nM ϵ subunit were performed for 0.5 min as described in Experimental Procedures. The rate of TMP release (s^{-1}) is the exonuclease rate. The amount of TMP released (nM) was determined by multiplying the concentration of each oligomer product by the number of TMPs released from the 3′ terminus to generate that product. The rate was then calculated by dividing the total TMP released by the enzyme concentration. The data were fit to a Michaelis−Menten curve [$k = k_{cat}S/(K_m + S)$] by nonlinear regression to yield a k_{cat} of 210 ± 23 and a K_m of 16 ± 6 μM.

(Abelbeck Software). Linear regression and standard errors were determined using SigmaPlot (Jandel Corp., v. 2.0).

RESULTS

Steady State Kinetics (dT_{10}). The ϵ subunit binds DNA poorly, requiring high concentrations to saturate the enzyme. Previous estimates indicated that the K_m value for the 3′ terminus of an oligonucleotide for ϵ subunit was greater than 10 μM (Maki & Kornberg, 1987). Steady state experiments were performed with high concentrations of dT_{10} (Table 1) in order to saturate the enzyme with 3′ termini. The activity of ϵ subunit using single-stranded DNA establishes an excision rate for the exonuclease without considerations of duplex DNA structure. The degradation of dT_{10} was measured at concentrations ranging from 6 to 90 μM DNA (Figure 1). From these data, the values for K_m of 16 ± 6 μM and k_{cat} of 210 ± 23 s^{-1} were determined. The steady state catalytic rate, k_{cat}, provides a lower limit on the magnitude of any first-order rate constants following the binding of substrate. From these data, the value of k_{cat}/K_m of 1.3×10^7 M^{-1} s^{-1} was calculated as a lower limit for the apparent second-order rate constant for substrate binding.

Inhibition of exonuclease activity by nucleoside monophosphates. The products of ϵ subunit activity are dNMP and DNA_{n-1}. The binding of the dNMP product was investigated by determining the inhibition constant K_i for the exonuclease. Degradation of dT_{10} by ϵ is inhibited by the addition of increasing concentrations of TMP to the standard steady state exonuclease assay. The reciprocal of the initial rate of nucleotide released plotted as a function of the reciprocal of the substrate concentration at three different concentrations of TMP is shown (Figure 2). These data indicate competitive inhibition; the K_i was determined to be 0.5 mM from a replot of the slopes as a function of inhibitor concentration (Figure 2, inset). The relatively high value of 0.5 mM for the inhibition constant indicates that TMP binds more weakly to the active site than does the dT_{10} substrate.

Pre-steady state kinetics (dT_{10}). In order to investigate individual steps in the exonuclease reaction, the pre-steady state kinetics were analyzed by a multiple-turnover experiment. The rate of dT_{10} degradation in the first and subsequent enzyme turnovers was measured at a high DNA concentration (85 μM; $5K_m$ determined under steady state conditions) with respect to the ϵ concentration (1.1 μM). The resulting monophasic linear curve has a slope of 310 ± 12 μM/s^{-1} for 1.1 μM ϵ, indicating a rate of 280 s^{-1} (Figure 3). The rate of the linear phase is slightly higher than the k_{cat} of 210 s^{-1} determined by steady state rate measurements using this substrate. The line generated from the data in Figure 3 has a *y*-intercept of 0.5 ± 0.21 μM. To determine if a burst of ϵ activity might be responsible for the non-zero y-intercept, kinetic simulations were performed using a two-step irreversible mechanism (Fersht, 1985; Johnson, 1992). Simulations using the calculated rate constants for a burst amplitude of 0.45 enzyme equivalent (0.5 μM 9mer/1.1 μM ϵ), $k_{cat} = 280$ s^{-1}, and the binding constant $K = 12$ μM do not generate curves that resemble the data from the multiple-turnover experiment in Figure 3 (not shown). Therefore, the data suggest that a slow product release step is not observed in the ϵ mechanism. The apparent lack of a burst

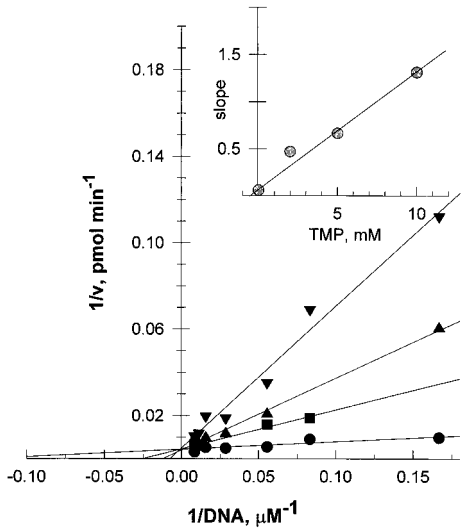

FIGURE 2: Inhibition of ϵ activity by TMP. The standard steady state exonuclease reaction mixtures (2.2 nM ϵ) were prepared as described in Experimental Procedures containing the indicated concentrations of dT_{10}. Reactions were performed for 0.5 min in the absence of TMP (●) and in the presence of 2 mM (■), 5 mM (▲), and 10 mM (▼) TMP. A replot of the slopes (inset) indicates a K_i of 0.5 mM.

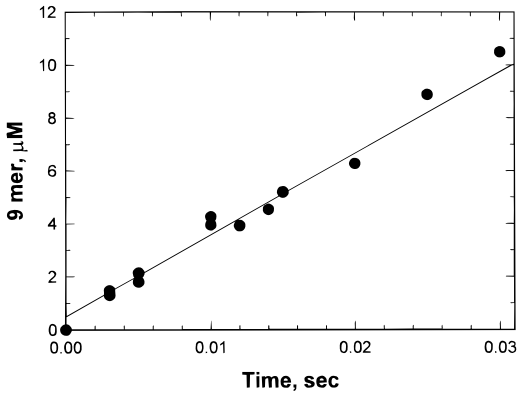

FIGURE 3: Monophasic kinetics of ϵ under multiple-turnover conditions. The ϵ subunit (1.1 μM) was mixed with dT_{10} (85 μM), reactions were quenched at the indicated times, and the amount of 9mer generated (●) was quantified. Duplicate reactions were performed at time points ≤10 ms. The data were fit to a straight line using linear regression.

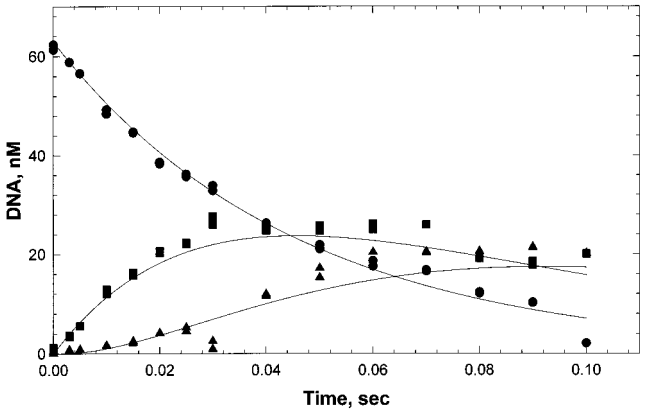

FIGURE 4: Single-turnover pre-steady state kinetics of dT_{10} degradation by ϵ. A rapid-quench reaction was performed as described in Experimental Procedures with 1.1 μM ϵ and 63 nM dT_{10}. The amount of 10mer (●), 9mer (■), and 8mer (▲) generated in the time course reaction was quantified. The symbols are the actual data collected, and the fitted curves were generated by kinetic simulation using 1.1 μM ϵ and 63 nM dT_{10} and the proposed mechanism and rate constants from Scheme 1. The degradation of dT_{10} fit a single-exponential curve with a rate of 21 ± 0.5 s^{-1}.

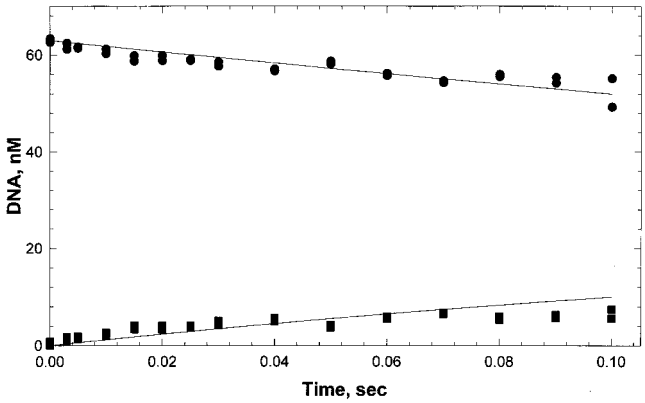

FIGURE 5: Pre-steady state kinetics of single-stranded GG20mer degradation by ϵ. The rapid-quench reaction was performed with 1.1 μM ϵ and 63 nM GG20mer. The amount of 20mer (●) and 19mer (■) generated in the time course reaction was quantified. The symbols are the actual data collected, and the fitted curves were generated by kinetic simulation using 1.1 μM ϵ and 63 nM GG20mer, the proposed mechanism from Scheme 1, and the rate constants $K = 12$ μM and $k_f = 30$ s^{-1}. The degradation of GG20mer fit a single-exponential curve with a rate of 1.6 ± 0.3 s^{-1}.

or an exponential phase corresponding to the first turnover indicates that there is no rate-limiting step after the bond cleavage step of the reaction.

Scheme 1 was proposed on the basis of the apparent lack of a rate-limiting step after bond cleavage and assuming a rapid equilibrium binding step (*K*). A series of single-turnover pre-steady state experiments was performed using a relatively high enzyme concentration (1.1 μM) and concentrations of dT_{10} ranging from 25 to 250 nM to test the validity of the proposed reaction mechanism. The degradation of the 10mer to generate 9mer and 8mer products was measured (Figure 4). The curves that best fit the data were generated by kinetic simulation using the distributive mechanism shown in Scheme 1 with $K = 12$ μM and $k_f = 280$ s^{-1}. Similar results were obtained for each of the DNA concentrations tested, supporting the proposed minimal mechanism in Scheme 1 for degradation of the dT_{10} oligonucleotide by ϵ subunit.

In order to test directly for a binding step that might be rate-limiting, degradation of dT_{10} was measured after pre-incubation with ϵ. Since ϵ activity requires a divalent cation for activity, a sample of ϵ, the dT_{10}, and all buffers were dialyzed against Chelex-100 to remove any bound metals. Using this procedure, ϵ exonuclease activity is diminished to <1% of that obtained upon addition of Mg^{2+}. The enzyme and DNA solutions were preincubated on ice for 15 min, and the $\epsilon-dT_{10}$ solution was loaded into one tube of the rapid-mixing apparatus. The reaction was initiated by mixing the $\epsilon-dT_{10}$ solution with an equal volume of 10 mM Mg^{2+} and quenched with 0.3 M EDTA. The degradation of the oligonucleotide by ϵ after preincubation with dT_{10} (not shown) followed the identical progress curve as observed when the enzyme and DNA were not preincubated (Figure 4). These results indicate that binding is not rate-limiting and that a rapid equilibrium step for binding is a valid model for the minimal mechanism.

ϵ activity on GG20mer. Single-stranded DNA is the preferred substrate for the exonuclease activity of ϵ subunit (Scheuermann & Echols, 1984; Maki & Kornberg, 1987). This preference for single-stranded DNA by ϵ raises questions about whether the active site of the proofreading exonuclease actually recognizes a single mismatch at the 3′ terminus of a partial duplex DNA. To determine the kinetic effect of DNA duplex structure on the activity of ϵ subunit, a series of oligonucleotides, complementary to the same 35mer oligonucleotide and containing all four bases, was synthesized (Table 1). Pre-steady state experiments were performed using the single-stranded 20mer (GG20mer) in order to determine the rate of degradation by ϵ subunit for comparison to that obtained for dT_{10} (Figure 5). The rate of degradation for the 20mer oligonucleotide was 13-fold slower than that for the dT_{10} substrate. Time course experiments performed under steady state conditions using 50 μM DNA also demonstrated a greater than 10-fold slower rate. The steady state rate for the dT_{10} substrate was 160 ± 20 s^{-1} and for the GG20mer was 10 ± 2.0 s^{-1} (not shown). The two nucleotides at the 3′ terminus of the GG20mer DNA (G-G) differ from those at the 3′ terminus of dT_{10} (T-T). To determine if sequence specific effects might contribute to the different degradation rates of these two oligonucleotides, the rate of exonucleolytic degradation of the homopolymer dG_{10} was compared to that of dT_{10}. The ϵ subunit degrades the dG_{10} with a steady state rate of 220 ± 70 s^{-1} (not shown), comparable to a value of 160 s^{-1} calculated for dT_{10} under similar conditions, suggesting that the slower rate of degradation for the GG20mer substrate is not due to sequence differences at the 3′ terminus.

Temperature Dependence of GG20mer Degradation. The presence of all four nucleotides in GG20mer makes it possible that structural elements such as internal base-pairing or base-stacking interactions might contribute to the slower rate of degradation of this DNA by ϵ. In order to investigate structural components of the DNA that might influence the degradation rate, steady state assays were performed at different temperatures (Figure 6). The rates of cleavage determined for dT_{10} are 1.5 ± 0.15 pmol/min at 37 °C and 1.3 ± 0.092 pmol/min at 28 °C. Thus, degradation of dT_{10} is relatively unaffected in this temperature range (Figure 6A). However, the rates of cleavage of the GG20mer are 1.2 ± 0.14 pmol/min at 37 °C and 0.6 ± 0.027 pmol/min at 28 °C (Figure 6B). At 37 °C, the rate of GG20mer degradation approaches that seen for dT_{10}. The dependence of the

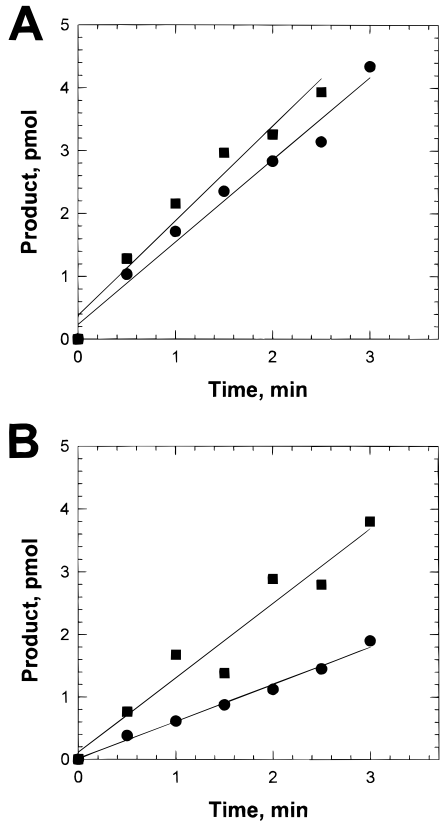

FIGURE 6: Effect of temperature on degradation of GG20mer and dT_{10} by ϵ. Exonuclease assays were carried out at 28 °C (●) and 37 °C (■) under steady state conditions using 1.4 nM ϵ and 40 nM dT_{10} (A) or 40 nM GG20mer (B) as substrate. The data were fit to a straight line using linear regression.

degradation rate of GG20mer by ϵ on temperature suggests that alterations in DNA structure at the 3′ terminus have an important effect on the activity of this enzyme. It is likely that a conformational change in the DNA is necessary for ϵ activity.

Pre-steady state kinetics (duplex DNA). The maximum excision rate by ϵ subunit is detected using the dT_{10} substrate. The physiological substrate of ϵ is not known. However, it is likely to be generated from duplex DNA containing a 3′ terminal mispair resulting from misincorporation by the α subunit. We designed a series of partial duplex DNA substrates containing zero to three mispaired nucleotides at the 3′ terminus (Table 1). These substrates allow measurement of the duplex structure effect on the exonuclease rate and the "minimal" size of the "melted" DNA for ϵ activity. The effect of DNA melting in the ϵ mechanism was quantified in single-turnover pre-steady state experiments by measuring excision rates for the 3′ terminal nucleotide using the four DNA substrates (Figure 7). The rates for each reaction increased as the number of mismatched nucleotides increased. To account for the slower rates observed using paired and mispaired duplex DNA substrates, an additional step was added in the reaction mechanism; the DNA melts or changes conformation to become a substrate for ϵ (Scheme 2). These results suggest that a DNA-melting step precedes excision by ϵ subunit and generation of single-stranded DNA at least three nucleotides in length is required. The apparent rates for the melting step using the duplex DNAs [$k_{app(n)}$] were derived from kinetic simulations of the data based on the mechanism in Scheme 2. The apparent rate for the

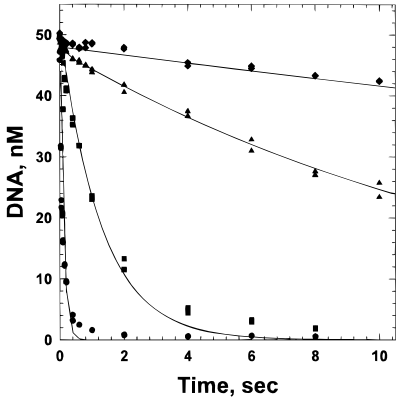

FIGURE 7: Comparison of exonuclease activities on partial duplex oligonucleotides. The ϵ subunit (1.1 μM) and primer−templates (50 nM) indicated in Table 1 were mixed, and reactions were quenched after the indicated times. The amount of full-length primer remaining was determined using the paired substrate (◆) and mispaired substrates containing a single (▲), double (■), and triple (●) mispair. The symbols are the actual data collected, and the fitted curves were generated by kinetic simulation using 1.1 μM ϵ and 50 nM DNA and the proposed mechanism in Scheme 2 with the rate constants $K = 12$ μM and $k_f = 280$ s^{-1}.

melting step using the paired DNA [$k_{app(0)}$] was determined to be 0.015 s^{-1}. For the mismatched DNAs, the apparent rates of $k_{app(1)} = 0.038$ s^{-1}, $k_{app(2)} = 0.78$ s^{-1}, and $k_{app(3)} = 100$ s^{-1} were determined. The models were initially unbiased, with the melting step occurring prior to or after formation of the enzyme−substrate complex. A better fit to the data was achieved when the melting step was modeled before substrate binding. The mechanism in Scheme 2 reflects this preference.

DISCUSSION

Exonucleolytic proofreading is necessary to maintain high levels of accuracy during replication. In *E. coli*, proofreading contributes at least 10^2-10^3-fold to the overall level of fidelity (Echols et al., 1983; Echols & Goodman, 1991). The universal importance of proofreading in DNA synthesis is shown by the fact that most DNA polymerases have an associated 3′ → 5′ exonuclease activity (Kornberg & Baker, 1992). This activity is usually contained within an integral domain of the polymerase polypeptide. In contrast, the exonuclease of pol III, ϵ subunit, is a distinct polypeptide that is active when separate from the remaining subunits of the pol III holoenzyme complex. In this paper, we describe the kinetics of the exonuclease reaction of ϵ subunit. Analysis of ϵ in the absence of the other pol III components has allowed us to distinguish characteristics of ϵ from those contributed by other subunits, particularly the polymerase subunit.

We have developed minimal kinetic schemes for the action of ϵ on single-stranded (Scheme 1) and duplex DNA (Scheme 2). These schemes and the indicated rate constants were used in simulations to model the experimental data. Initial steady state experiments using the single-stranded DNA, dT_{10}, demonstrated that substrate binding was saturable at relatively high DNA concentrations. These steady state experiments provide accurate determinations $K_m = 16 \pm 6$ μM and $k_{cat} = 210 \pm 23$ s^{-1}. The steady state values are in agreement with the pre-steady state values modeled as dissociation constant (K_d) = 12 μM and $k_f = 280$ s^{-1}. These results are consistent with a simple two-step reaction

mechanism for degradation of dT_{10} by ϵ subunit. The first step is a saturable formation of the $\epsilon-dT_{10}$ complex that is followed by conversion of dT_{10} to dT_9 in the chemical step (Scheme 1). A pre-steady state burst was not apparent in the analysis of ϵ, although it is possible that under different reaction conditions such a burst might be revealed. The lack of a pre-steady state burst of product in an enzyme-catalyzed reaction suggests that either the chemical step or a step prior to chemistry is rate-limiting (Johnson, 1992). The pre-steady state analysis indicates that the K_d for the $\epsilon-$DNA complex is relatively high. It is unlikely that this high K_d value can be attributed to a slow association rate constant because preincubation of ϵ with dT_{10} does not result in a burst of product under pre-steady state conditions. Furthermore, dissociation of ϵ from DNA is not rate-limiting since a biphasic curve was not observed in the multiple-turnover experiments. Thus, the chemical step is most likely the rate-limiting step in the ϵ-catalyzed reaction.

The K_m values for exonuclease activity by the pol III core and the $\alpha-\epsilon$ complex using mispaired DNA are 0.14−0.46 μM (Brenowitz et al., 1991; Maki & Kornberg, 1987). These substantially lower K_m values, relative to a K_m value greater than 10 μM for ϵ subunit alone (Maki & Kornberg, 1987), suggest that the α or θ subunit (or both) contributes to substrate binding by ϵ subunit in the enzyme complex. The inhibition studies with TMP show a K_i of 0.5 mM, supporting the idea of weak product binding by ϵ subunit. The K_i values for TMP using pol III core and $\alpha-\epsilon$ have not been determined. However, the K_i value for DNA pol I is 0.132 mM (Que et al., 1978), almost 4-fold lower than that for ϵ. In addition, the K_m value for DNA of 0.08 μM for the exonuclease activity of DNA pol I is 250 times lower than that for ϵ. Our results suggest that ϵ subunit binds DNA poorly as might be predicted for a catalytic activity within the holoenzyme complex that plays a passive role in the DNA synthesis process.

The structure of DNA plays a key role in the observed rate of cleavage by ϵ subunit. Using duplex DNA, increased reaction rates for ϵ and pol III core are detected with increased temperatures indicating the importance of the DNA conformation in the exonuclease reaction (Brenowitz et al., 1991). The temperature effect detected using the GG20mer oligonucleotide (Figure 6B) might be attributed to "duplex-like" structure in the oligomer. The activity of ϵ using the GG20mer can be described by Scheme 2. In this mechanism, a step is included for the conversion of DNA that is not substrate for ϵ to the substrate DNA* as a contribution to the overall rate. This rate constant (k_{app}) is temperature dependent as might be expected for DNA duplex melting. At 37 °C, the reaction rate using GG20mer approaches that using dT_{10}, suggesting the removal of duplex-like structure in GG20mer. Thus, a kinetic contribution related to DNA melting can be determined.

Analysis of the ϵ reaction supports the melting hypothesis for exonucleolytic proofreading (Brutlag & Kornberg, 1972; Bessman & Reha-Krantz, 1977). The rate of degradation of single-stranded DNA is greater than that of paired DNA for proofreading exonucleases. Furthermore, excision of the 3′ terminal nucleotide using duplex DNAs containing one to three mispairs indicates that at least three nucleotides of single-stranded DNA are required to achieve the maximum excision rate. The ϵ subunit degrades dT_4 oligomers rapidly from dT_4 to dT_2 and slowly from dT_2 to dT (Perrino et al., 1994, and unpublished results). The only clear oligomer length effect on ϵ cleavage that has been detected is of the dinucleotide to the monomer, further supporting a minimal substrate length for ϵ of three nucleotides. The preference for single-stranded DNA suggests that the rate-limiting step in exonucleolytic proofreading is melting of duplex DNA and not cleavage of the 3′ terminal nucleotide. In the case of T7 DNA polymerase, duplex DNA binds to the polymerase active site and exonuclease activity is attributed to the difference between polymerization from a mispaired 3′ terminus (k_{pol}) and transfer to the exonuclease active site ($k_{pol-exo}$) (Donlin et al., 1991). For T4 DNA polymerase, the rate-limiting step has been proposed to be a DNA-melting step (Capson et al., 1992). The precise mechanism of DNA melting in polymerase-associated exonucleases has not been clearly established. In studies using ϵ subunit, there is no polymerase active site that might bind and compete for DNA. Therefore, our results can be clearly attributed to the requirement of DNA melting. Furthermore, our kinetic analysis suggests that the physiologically relevant substrate for the ϵ subunit within the holoenzyme complex is single-stranded DNA at least three nucleotides in length. The mechanistic details of the ϵ exonuclease with its DNA substrate within the holoenzyme complex are likely more complex than the minimal schemes used here to examine the exonuclease activity alone.

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