Mutants Affecting Nucleotide Recognition by T7 DNA Polymerase

Maureen J. Donlin‡ and Kenneth A. Johnson*

Department of Biochemistry and Molecular Biology, 106 Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT: Analysis of two mutations affecting nucleotide selection by the DNA polymerase from bacteriophage T7 is reported here. Two conserved residues (Glu480 and Tyr530) in the polymerase active site of an exonuclease deficient (exo⁻) T7 DNA polymerase were mutated using site-directed mutagenesis (Glu480-Asp and Tyr530-Phe). The kinetic and equilibrium constants governing DNA binding, nucleotide incorporation, and pyrophosphorolysis were measured with the mutants E480D(exo⁻) and Y530F(exo⁻) in single-turnover experiments using rapid chemical quench-flow methods. Both mutants have slightly lower K_d values for DNA binding compared to that of wild-type(exo⁻). With Y530F(exo⁻) the ground state nucleotide binding affinity was unchanged from wild-type for dGTP and dCTP, was 2-fold lower for dATP and 8−10-fold lower for dTTP binding. With E480D(exo⁻), the binding constants were 5−6-fold lower for dATP, dGTP, and dCTP and 40-fold lower for dTTP binding compared to those constants for wild-type(exo⁻). The significance of a specific destabilization of dTTP binding by these amino acids was examined using a dGTP analog, deoxyinosine triphosphate, which mimics the placement and number of hydrogen bonds of an A:T base pair. The K_d for dCTP opposite inosine was unchanged with wild-type(exo⁻) (197 μM) but higher with Y530F(exo⁻) (454 μM) and with E480D(exo⁻) (1 mM). The K_d for dITP was the same with wild-type(exo⁻) (180 μM) and Y530F(exo⁻) (229 μM), but significantly higher with E480D(exo⁻) (3.2 mM). These data support the suggestion that E480 selectively stabilizes dTTP in the wild-type enzyme, perhaps by hydrogen bonding to the unbonded carbonyl. Data on the incorporation of dideoxynucleotide analogs were consistent with the observation of a selective stabilization of dTTP by both residues. Pyrophosphorolysis experiments revealed that neither mutation had a significant effect on the chemistry of polymerization. The fidelity of the mutants were examined in misincorporation assays. Both E480D(exo⁻) and Y530F(exo⁻) showed saturation kinetics with the wrong nucleotide, with binding constants of 1−3 mM compared to the estimated binding affinity of 6−8 mM with wild-type-(exo⁻). Accordingly, both mutants showed slightlty lower selectivity against misincorporation. Taken together, these results indicate that E480 and Y530 each contribute to ground state nucleotide binding and suggest that the E480 may serve to specifically stabilize the incoming dTTP of A:T base pairs to compensate for the fewer hydrogen bonds compared to G:C base pairs.

The mechanism of polymerization catalyzed by T7 DNA polymerase, including its mechanism for maintaining fidelity and the mechanism of its associated 3′ → 5′ proofreading exonuclease activity have been determined (Patel et al., 1991; Wong et al., 1991; Donlin et al., 1991). T7 DNA polymerase forms the core of the relatively simple replication complex of bacteriophage T7 (Richardson, 1983) and is the best simple model for examining the kinetics of DNA replication. We are interested in extending the kinetic analysis of T7 DNA polymerase to a structure-function analysis of its polymerase domain; the work done in solving the kinetic mechanism of the wild-type enzyme will serve as the starting point for the studies presented in this paper.

T7 DNA polymerase is functionally well-characterized, but little information is available about its structure. We can begin to probe structure-function relationships without a structure for the enzyme based upon sequence homology. Amino acids that are strictly conserved a series of related enzymes are likely to be structurally or functionally important in catalysis. The conserved amino acids can be altered by site-directed mutagenesis and the resulting mutant proteins analyzed using pre-steady-state kinetics. Pre-steady-state kinetic analysis allows definition of the role for conserved residues within the context of the kinetic mechanism of wild-type T7 DNA polymerase.

T7 DNA polymerase shares significant sequence homology with DNA Polymerase I from *E. coli* (Pol I) (Argos et al., 1986; Ollis et al., 1985), as well as with the DNA polymerases from phage T5 (Leavitt & Ito, 1989), phage SPO2 (Delarue et al., 1990), *Thermus aquaticus* (Lawyer et al., 1989), and *Staphylococcus pneumoniae* (Lopez et al., 1989). The sequences of these six enzymes have been aligned to reveal five well conserved regions within the C-terminal domain of each polymerase (Delarue et al., 1990). Three of the five conserved regions of the Pol I-like DNA polymerases correspond to the most highly conserved regions among the Pol α-like polymerases (Wang et al., 1989); this suggests an important role for these regions in structure or catalysis and a functional homology between similar domains among different families of DNA polymerases.

Two amino acids were altered in the T7 DNA polymerase in the present study. The first, Tyr530, is strictly conserved among all the DNA-dependent DNA polymerase families as well as among the DNA-dependent RNA polymerases,

‡ Current Address: Department of Plant Biology, University of California−Berkeley, Berkeley, CA 94720.

* Author to whom correspondence should be addressed.

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Table 1: Oligonucleotides Used for Kinetic Analysis

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25/36A-mer
5'-GCCTCGCAGCCGTCCAACCAACTCA
   CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5'

25/36T-mer
5'-GCCTCGCAGCCGTCCAACCAACTCA
   CGGAGCGTCGGCAGGTTGGTTGAGTTGGTCTTGTTT-5'

25/36C-mer
5'-GCCTCGCAGCCGTCCAACCAACTCA
   CGGAGCGTCGGCAGGTTGGTTGAGTCGGTCTTGTTT-5'

25/36G-mer
5'-GCCTCGCAGCCGTCCAACCAACTCA
   CGGAGCGTCGGCAGGTTGGTTGAGTGCGTCTTGTTT-5'

25/36I-mer
5'-GCCTCGCAGCCGTCCAACCAACTCA
   CGGAGCGTCGGCAGGTTGGTTGAGTIGGTCTTGTTT-5'

30/60-mer
5'-GCCTCGCAGCCGTCCAACCAACTCTACCCT
   CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGGGATTGAAGTAGGTACACCAGTCGTTTA-5'
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suggesting a crucial role for this residue in polymerization (Delarue, 1990). Phe was chosen to replace Tyr530 to minimize the structural perturbation, while removing the hydroxyl group which might be involved in nucleotide binding. The second mutation, Glu480-Asp, was chosen because a strictly conserved Glu residue located near the DNA binding cleft could be involved in either binding the Mg^{2+} complexed to the dNTP or in a direct interaction with the dNTP base structure. Mutating the Glu480 to an Asp leaves the carboxyl group intact but shifts it by approximately 1 Å, presumably away from the active site (Raines et al., 1986). In addition, both of the homologous residues in Pol I, Tyr766 and Glu710, have been mutated and the mutant proteins have been analyzed (Carroll et al., 1991; Polesky et al., 1992), so it should be possible to determine if functional homology is conserved between the two polymerases.

MATERIALS AND METHODS

Nomenclature. Mutations were introduced into an exonuclease deficient mutant of T7 pol (exo$^-$) made by alteration of Glu at position 5 to Ala and Asp at position 7 to Ala (Patel et al., 1991; Wong, 1990). This clone was originally named pGA1−14; it will be called pG5X (G5 for gene 5 of phage T7 and X, for exonuclease deficient). The clones containing the two mutants made in the exo$^-$ background, Tyr at position 530 to Phe and Glu at position 480 to Asp, were named pG5X(Y530F) and pG5X(E480D), respectively. The mutant proteins will be referred to as E480D(exo$^-$) and Y530F(exo$^-$). The T7 DNA polymerase used throughout this study was the exonuclease deficient form and will be hereafter referred to wild-type(exo$^-$) to distinguish it from wild-type T7 DNA polymerase having the full 3′ → 5′ exonuclease activity.

Oligonucleotides. Oligonucleotides for mutagenesis (Table 1) and DNA sequencing were synthesized on a Millipore 7500 DNA synthesizer and were received crude and deprotected in an ammonium hydroxide solution. The oligonucleotides were dried under vacuum in a speed-vac and the dried pellets were resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Residual contaminants from the synthesis were removed by extraction with phenol and chloroform.

Mutagenesis Procedure. The mutagenesis procedure used to generate the Y530F(exo$^-$) and E480D(exo$^-$) mutants was based on that of Zoller and Smith (1982) as modified by Kunkel (1985) to increase the mutation frequency by providing a selection against the wild-type(exo$^-$) parental molecules. pG5X was propagated in an dut$^-$ ung$^-$ *Escherichia coli* strain, CJ236, to generate plasmid DNA containing uracils in place of thymidine residues. Single-stranded molecules were produced by addition of the helper phage, M13KO7. The mutagenic oligos were annealed and the second strand was synthesized in vitro using T4 DNA polymerase in 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 2 mM DTT with 1 mM ATP and 0.5 mM each of the four dNTPs. The resulting heteroduplex DNA molecules were transformed into a dut$^+$ ung$^+$ *E. coli* strain, JM109, to provide selection for the progeny molecules. Mutations in the progeny pG5X DNA were identified by sequencing the entire gene.

Expression and Purification of the Mutant Proteins. The plasmids expressing the mutant proteins were transformed into in a thioredoxin-deficient strain of *E. coli*, A179, containing the plasmid pGP1 using the method of Hanahan (1983). High expression of the mutant proteins was accomplished as described (Tabor et al., 1987; Wong et al., 1991). Crude lysates were made by suspending the cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT). The lysis mixture was frozen in liquid N_2 and thawed at 37 °C until just liquid; this was repeated twice to ensure complete lysis. NaCl was added to a final concentration of 500 mM, and the mixture was incubated on ice for 90 min. Cellular debris was removed by centrifugation. The supernatant was removed and saved for use in the thioredoxin-dependent polymerization assay. Both mutant proteins were purified to homogeneity according to Wong et al. (1991).

Thioredoxin-Dependent DNA Polymerization Assay. Lysis supernatant (10 μL) was mixed with 20 μL of 5× T7 reaction buffer (100 mM Tris-HCl, pH 7.5, 63.5 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM DTT, and 0.5 mg/mL BSA) 10 μL of a solution of all four dNTP (0.3 mM each), 5 μL of [α-^{32}P]-dCTP (50 μCi), 5 μL of thioredoxin (500 μM), and 30 μL of H_2O. A small aliquot (10 μL) was removed as a zero point, and denatured calf-thymus DNA (20 μL, 3 mg/mL) was added to initiate DNA synthesis. Aliquots were removed at intervals from 15 s to 15 min and quenched by adding EDTA to final concentration of 250 mM. The aliquots were spotted on DE81 filters to monitor the incorporation of [α-^{32}P]dCTP into the calf thymus DNA. The filters were washed in 0.3 M ammonium formate (pH 8.0) to remove free nucleotides and the incorporated radioactivity was measured by liquid scintillation counting. Control reactions to measure background DNA polymerization activity were done under identical conditions, but without the addition of thioredoxin.

Oligonucleotides. The oligonucleotide primer/templates used in the kinetic assays (Table 1) are designated as follows. The primer length (25 nt) is listed first, followed by the template length (36 nt) and a letter designating the nucleotide at position 26 of the template (which dictates the first nucleotide to be incorporated). The primer is identical to that used for determining the mechanism of polymerization for the wild-type(exo$^-$) T7 DNA polymerase (Patel et al., 1991). The template varies by 1−2 nucleotides to allow incorporation of different dNTPs in single nucleotide incorporation assays. The oligonucleotides were synthesized on Millipore 7500 DNA synthesizer and were received crude

and deblocked in ammonium hydroxide. The oligonucleotides were purified by electrophoresis through a denaturing gel (18% acrylamide, 1.5% bisacrylamide, and 8 M urea in TBE buffer). The major band was visualized by UV shadowing, excised, and electroeluted in an Elutrap (Schleicher and Schuell) in TBE buffer. Concentrations of the purified oligonucleotides were determined by UV absorbance at 260 nm with the following extinction coefficients: 25-mer, ϵ = 249 040; 36-mer, ϵ = 377 000; 30-mer, ϵ = 286 310; and 60-mer, ϵ = 667 030 L cm^{-1} mol^{-1}.

Reconstitution of the T7 DNA Polymerases. T7 DNA polymerases were reconstituted immediately prior to use. Thioredoxin was added to standard T7 buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and 50 mM NaCl), and then the gene 5 protein was added to obtain a final molar ratio of 1:20 (gene 5 protein: thioredoxin).

5′-Radiolabeling of Primer-Templates. Single-stranded primer (25-mer) was 5′-radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maniatis et al. (1982) for protruding 5′ termini, with the following modifications. The labeling mix was incubated at 37 °C for 30 min followed by a 2-min incubation at 90 °C to denature the kinase. While the labeling mix was still hot, single-stranded template was added in slight excess, the reaction was vortexed and allowed to incubate at room temperature for 20 min to allow the strands to anneal.

3′-Radiolabeling of 25/36A-mer. Duplex 25/36A-mer (1 μM) was preincubated in standard T7 buffer with exo$^-$ T7 pol (100 nM). The reaction was initiated by adding 100 μCi of [α-^{32}P]dTTP in 20 μM dTTP, and this mixture was incubated at 20 °C for 5 min. The reaction was terminated by extraction with phenol:chloroform (1:1). The aqueous phase was removed and passed over a Bio-spin 30 column (Bio-Rad Laboratories) to remove unincorporated nucleotides.

Rapid-Chemical-Quench Flow Experiments. Rapid-quench experiments were performed by using an apparatus designed by K. A. Johnson (1986) and built by KinTek Instruments Corp. (State College, PA). Because of the absolute requirement of Mg^{2+} for T7 DNA polymerase enzymatic activity, the enzyme and DNA could be preincubated together without Mg^{2+} and the reaction initiated by the addition of Mg^{2+}. No differences were observed in either the rate or amplitude of the burst if the Mg^{2+} was preincubated with the enzyme−DNA complex prior to initiating the reaction by the addition of nucleotide in experiments performed with wild-type (exo$^-$) T7 pol. The concentrations of enzyme and DNA listed for each experiment are the concentrations after mixing and during the enzymatic reactions. Reactions were quenched with EDTA at a final concentration of 0.3 M at time intervals from 3 ms to several seconds. Reactions were done at 20 °C unless otherwise noted.

Misincorporation Assays. Experiments to determine the rate of incorporation of a mispaired nucleotide were done in standard T7 buffer at room temperature (23−26 °C) with 5′-radiolabeled DNA in greater than 10-fold excess over enzyme. The enzyme and DNA were preincubated in the absence of Mg^{2+} and the reaction initiated by the addition of the incorrect nucleotide and Mg^{2+}. Aliquots were removed at time intervals from 5 to 60 s and the reactions quenched in EDTA at a final concentration of 250 mM.

Product Analysis. Reaction products from 5′-radiolabeled substrates were analyzed by electrophoresis through denaturing gels (16% polyacrylamide, 8 M urea in TBE buffer) and were quantified by excising the bands and the counting the radioactivity in each band by liquid scintillation. Reaction products from 3′-labeled substrates were analyzed on PEI-cellulose thin-layer chromatography plates developed in 0.3 M KH_2PO_4 (pH 3.4) and quantified by excising the bands and counting the radioactivity in each band by liquid scintillation.

Data Analysis. The data were fit to single mathematical expressions by nonlinear regression using the program RS/1 (BBN Software Products Corp., Cambridge MA), run on a Digital Microvax II computer.

RESULTS

Both E480D(exo$^-$) and Y530F(exo$^-$) mutants of T7 pol were shown to have thioredoxin-dependent DNA polymerization activity in crude extracts. The mutants were therefore examined in a processive DNA synthesis assay to determine, qualitatively, their differences with wild-type(exo$^-$). The role of E480 and Y530 in forward polymerization were then defined in detail using pre-steady-state experiments conducted on a rapid-quench apparatus to measure the rapid rate of DNA synthesis.

The substrates used in these studies were synthetic DNA primer/templates of defined length and sequence (Table 1). These oligonucleotides were gel-purified and were designed to allow single nucleotide incorporation.

Processive DNA Synthesis. Processive DNA polymerization was examined for the Y530F(exo$^-$) and E480D(exo$^-$) mutants and wild-type(exo$^-$) T7 pol by mixing the enzyme with a 5′-labeled 30/60-mer template (Table 1) in the presence of the next three correct nucleotides to extend the primer 17 bases to a 47-mer. The enzyme (50 nM) and DNA (100 nM) were preincubated and then mixed with the three dNTPs (100 μM each). Time points were taken from 2 ms to 4 s, by quenching the reaction in 0.3 M EDTA. The reaction products were resolved by denaturing polyacrylamide electrophoresis and detected by autoradiography; one such autoradiograph is shown in Figure 1.

In this experiment, both the processivity and rates of polymerization can be evaluated quantitatively because it was performed with DNA in excess (Patel et al., 1991; Kati et al., 1992). Qualitatively, higher processivity results in full length products and starting material with few intermediate length DNA products. Lower processivity is a function of extension versus enzyme dissociation from the DNA and results in the accumulation of products of intermediate length. Wild-type(exo$^-$) T7 pol is the most processive of the three enzymes. The E480D(exo$^-$) mutant shows an apparent reduced rate of polymerization as well as lower processivity, and the Y530F(exo$^-$) mutant shows less processivity than does wild-type(exo$^-$), but the rate of polymerization does not appear to be significantly reduced overall. The mechanism of both mutants was investigated in detail using single nucleotide incorporation assays.

Active Site Titration of E-DNA. The equilibration of the T7 DNA polymerase−DNA complex is slow relative to the forward rate of polymerization, so it is possible to quantify the formation of the E-DNA complex by measuring the amplitude of the burst of polymerization involving the

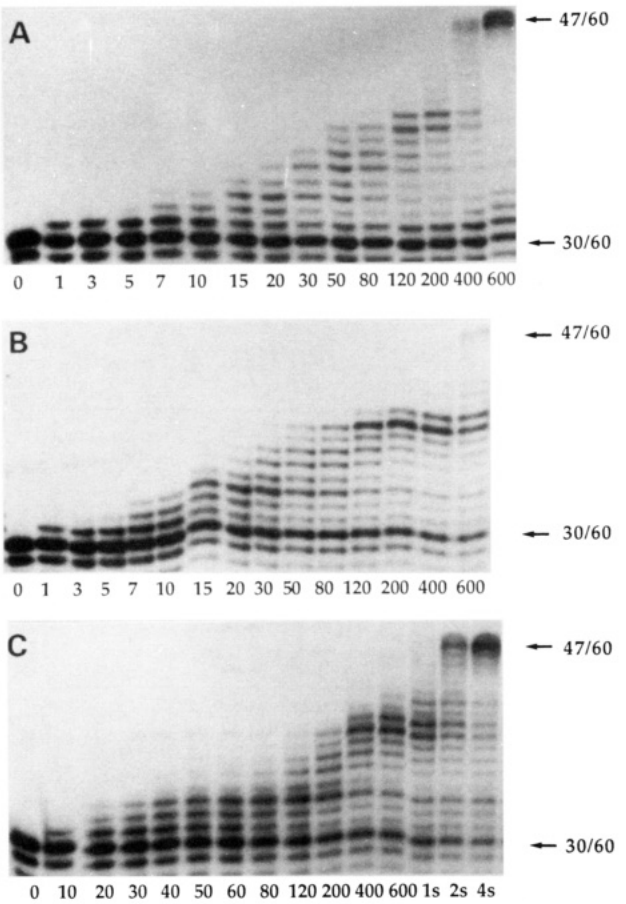

FIGURE 1: Processivity of DNA synthesis. The experiment was conducted by mixing the T7 DNA polymerases [wild-type(exo$^-$) (A); Y530F(exo$^-$) (B), or E480D(exo$^-$) (C), (50 nM)] and 5′-^{32}P-labeled 30/60-mer (100 nM) with dTTP, dATP, and dCTP (100 μM each) in T7 buffer containing magnesium. The reactions were quenched in 0.3 M EDTA and analyzed by denaturing gel electrophoresis. The times listed under each lane in the autoradiograph are in milliseconds unless otherwise noted.

addition of a single correct nucleotide. Titration of the active site is accomplished by measuring the DNA concentration dependence of the pre-steady-state burst amplitude. As with the wild-type(exo$^-$) T7 pol (Patel et al., 1991), both the E480D(exo$^-$) and Y530F(exo$^-$) mutants form a stable complex with the DNA. The titration was carried out at fixed enzyme concentration (50 nM) and increasing concentration of the substrate 25/36A-mer (Table 1). The enzyme and DNA were preincubated in the absence of Mg^{2+} and the reaction was initiated with the addition of the next correct nucleotide (100 μM dTTP) and Mg^{2+}. The amount of product (26/36-mer) was measured at each DNA concentration. The burst amplitude was plotted against the 25/36A-mer concentration and fit to the quadratic equation (data not shown). This measured the K_d for formation of productive complex between 25/36A-mer and the two mutants. The K_d was 9 nM for Y530F(exo$^-$) and 6 nM for E480D(exo$^-$), compared to 18 nM for wild-type T7 pol (Patel et al., 1991) and 16 nM for exo$^-$ T7 pol (Wong, 1990).

K_d for Deoxynucleotides. The equilibrium dissociation constant for each dNTP was determined by measuring the concentration dependence of the rate of formation of 26/36-mer at a fixed E-DNA concentration. The rates of single nucleotide incorporation were plotted against the dNTP concentrations and fit to a hyperbola to give a measurement of the K_d for the dNTP and the maximal velocity of

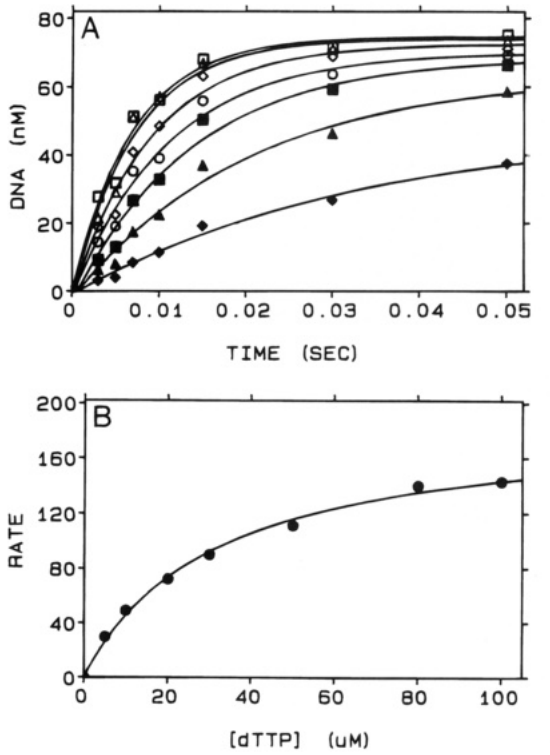

FIGURE 2: dTTP concentration dependence of the pre-steady-state burst rate: wild-type(exo$^-$) at 10 °C. (A) Wild-type(exo$^-$) T7 pol (50 nM) and 5′-labeled 25/36A-mer (100 nM) were preincubated, mixed with increasing concentrations of Mg^{2+}•dTTP to start the reactions, and incorporation of dTMP was analyzed by denaturing gel electrophoresis. The dTTP concentrations were 5 μM (◆), 10 μM, (▲) 20 μM (■), 30 μM (○), 50 μM (◇), 80 μM (△), and 100 μM (□). The solid lines represent the fit of the data to a single exponential. (B) the pre-steady-state rates were plotted against the dTTP concentrations and fit to a hyperbola to determine the K_d and maximum incorporation for dTTP with wild-type(exo$^-$).

polymerization for each nucleotide (Figures 2−4 and Table 1). At 10 °C, the K_d for dTTP with wild-type(exo$^-$) was 31 ± 3 μM, and the maximal velocity was 188 ± 7 s^{-1} (Figure 2). For Y530F(exo$^-$) the K_d was 139 ± 22 μM with a maximal velocity of 219 ± 13 s^{-1} (Figure 3). For E480D(exo$^-$), the K_d for dTTP was 760 ± 20 μM with a maximal rate of 71 ± 1 s^{-1} (Figure 4).

Using the alternate templates listed in Table 1 to allow for single nucleotide incorporation, the K_d values for the other three nucleotides were measured with both mutants and wild-type(exo$^-$) (Table 2). The K_d values for the other three dNTPs with Y530F(exo$^-$) mutant were measured at 10 °C because of the inaccuracies in measuring the rapid rates of incorporation seen at 20 °C. To control for the temperature dependence of the reactions, the K_d for dTTP with wild-type(exo$^-$) and both mutants were also measured at 20 °C; the results are listed in Table 2. With the E480D(exo$^-$) mutant, the maximum rates of incorporation were sufficiently slow to allow accurate measurement at 20 °C.

The Y530F(exo$^-$) mutant exhibits essentially unchanged K_d values for dGTP and dCTP compared to those of the wild-type(exo$^-$) T7 pol. The K_d for dATP is slightly higher and for dTTP the K_d is significantly higher than that measured with wild-type(exo$^-$) at either temperature. These results suggest that the Y530 residue may selectively stabilize A:T base pairs, possibly by specifically stabilizing dTTP binding. The loss of the hydroxyl group of Y530 results in lower affinity only for dTTP.

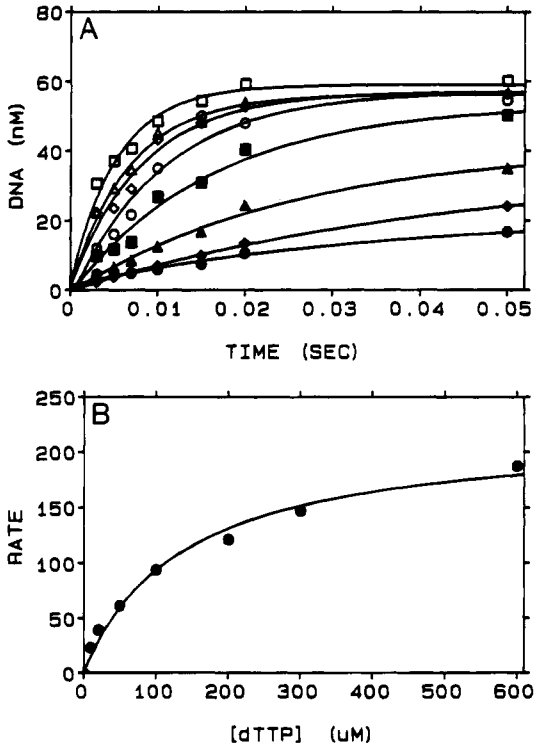

FIGURE 3: dTTP concentration dependence of the pre-steady-state burst rate: Y530F(exo$^-$) at 10 °C. (A) Y530F(exo$^-$) T7 pol (50 nM) and 5′-labeled 25/36A-mer (100 nM) were preincubated, mixed with increasing concentrations of Mg^{2+}•dTTP to start the reactions, and incorporation of dTMP was analyzed by denaturing gel electrophoresis. The dTTP concentrations were 5 μM (●), 10 μM (◆), 50 μM (▲), 100 μM (□), 200 μM (○), 300 μM (◇), 600 μM (△), and 800 μM (□). The solid lines represent the fit of the data to a single exponential. (B) The pre-steady-state rates were plotted against the dTTP concentrations and fit to a hyperbola to determine the K_d and maximum incorporation for dTTP with Y530F(exo$^-$).

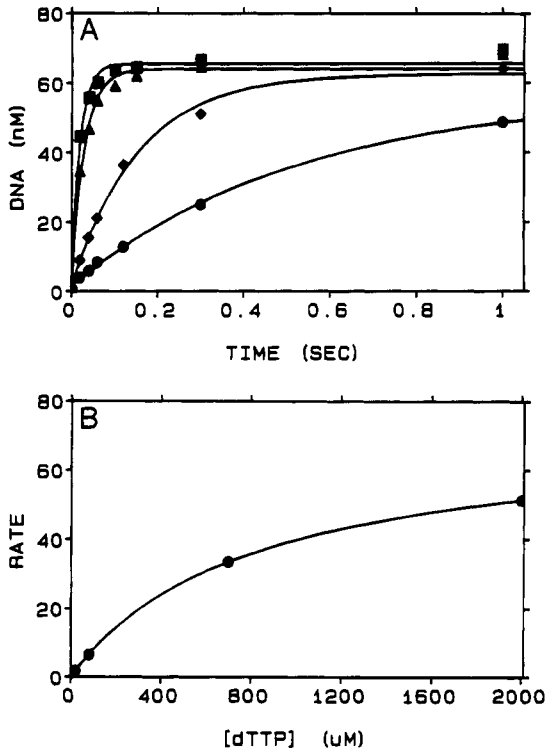

FIGURE 4: dTTP concentration dependence of the pre-steady-state burst rate: E480D(exo$^-$) at 10 °C. (A) The E480D(exo$^-$) T7 pol (50 nM) and 5′-labeled 25/36A-mer (100 nM) were preincubated, mixed with increasing concentrations of Mg^{2+}•dTTP to start the reactions, and incorporation of dTMP was analyzed by denaturing gel electrophoresis. The dTTP concentrations were 20 μM (●), 80 μM (◆), 700 μM (▲), and 2000 μM (■). The solid lines represent the fit of the data to a single exponential. (B) The pre-steady-state rates were plotted against the dTTP concentrations and fit to a hyperbola to obtain the K_d and maximal incorporation rate for dTTP with E480D(exo$^-$).

It is also apparent from these results that the E480 residue plays an important role in binding and/or stabilization of all four nucleotides. The slightly higher K_d observed for dATP and much higher K_d for dTTP relative to the other dNTPs suggest that this amino acid also contributes toward selective stabilization of the incoming dTTP of an A:T base pair.

It is surprising that the inherent differences expected in binding energy between A:T and C:G base pairs have not been observed with wild-type enzyme; in fact, the binding constants for all four dNTPs are nearly identical with wild-type(exo$^-$) T7 pol (Wong et al., 1991). Therefore, the A:T base pairs must be selectively stabilized at the active site to compensate for one less hydrogen bond in the A:T base pair. Both mutations that we examined selectively weakened the binding of A:T base pairs at the polymerase site, perhaps by removing the groups involved in selective stabilization. This hypothesis was examined in further detail using inosine, an analog of guanosine.

Inosine Analog. The data for the mutants presented thus far suggest that both the Y530 and E480 residues are involved in stabilizing dTTP binding relative to the other three deoxynucleotides. One possible site of interaction is the carbonyl oxygen at position C-2 of thymidine. When paired with dATP, this carbonyl oxygen does not interact with any portion of the adenine ring and so may interact with an amino acid within the polymerase active site.

To test this hypothesis, the interaction of a guanosine analog, inosine, was examined. With inosine in the template, incorporation of a cytosine should mimic thymidine incorporation opposite an adenine in the sense of having only two hydrogen bonds in the base pair and a functional group

Table 2: Kinetic Constants for Deoxynucleotide Incorporation[a]

	K_d		
dNTP	wild-type (μM)	Tyr530-Phe (μM)	Glu480-Asp (μM)
dTTP	18 ± 3[b]	169 ± 21	720 ± 13
dTTP	[31 ± 3]	[139 ± 22]	[760 ± 20]
dATP	18 ± 3	[28 ± 3]	137 ± 25
dGTP	[18 ± 3][c]	[17 ± 3]	110 ± 5
dCTP	17 ± 2	[14 ± 5]	106 ± 6
dITP:C	[180 ± 38]	229 ± 94	3.2 ± 0.3
dCTP:I	197 ± 38	454 ± 45	1.0 ± 0.25
ddTTP	43 ± 2	45 ± 7	313 ± 40
ddATP	21 ± 3	92 ± 12	900 ± 35
	k_{cat}		
dNTP	wild-type (s^{-1})	Tyr530-Phe (s^{-1})	Glu480-Asp (s^{-1})
dTTP	297 ± 10	279 ± 38	205 ± 5
dTTP	[188 ± 7]	[219 ± 13]	[71 ± 1]
dATP	280 ± 4	[167 ± 3]	125 ± 8
dGTP	[246 ± 12][c]	[198 ± 3]	161 ± 2
dCTP	447 ± 30	[148 ± 11]	125 ± 8
dITP:C	[219 ± 15]	140 ± 15	24 ± 2
dCTP:I	400 ± 30	293 ± 30	141 ± 13
ddTTP	46 ± 2	6.2 ± 0.2	1.5 ± 0.7
ddATP	86 ± 3	52 ± 2	5.2 ± 0.9

[a] Constants shown in brackets were determined at 10 °C; all others were determined at 20 °C. [b] From Patel et al. (1991). [c] From Kati and Johnson (1994).

at position C-2 of the base which must be satisfied by H-bonding with something other than the template base. If this is the case, one might expect a larger K_d for dCTP with both mutants relative to its K_d with wild-type(exo$^-$) T7 pol. The incorporation of dITP opposite a template cytosine was also measured so as to distinguish between the effects of the mutations and the effects of an altered substrate. The template used for dCTP incorporation has an inosine in position 26 (26I-mer; Table 1).

With cytosine in the template, the K_d for dITP was measured with wild-type(exo$^-$) enzyme and both mutants to give the results in Table 2. Both wild-type and Y530F(exo$^-$) have approximately the same affinity and rate of incorporation for dITP under these conditions, although the affinity was approximately 10-fold weaker than that for dGTP with the same template. The K_d for dITP with the E480D(exo$^-$) mutant was much higher (3 mM versus 100 μM for dGTP) with a much slower rate of incorporation compared to wild-type(exo$^-$). This may reflect a structural perturbation of the alternate substrate to which this mutant is more sensitive than is Y530F(exo$^-$) or wild-type(exo$^-$) T7 pol.

With inosine in the template, the K_d for dCTP was measured with wild-type(exo$^-$) enzyme and both mutants and the results are listed in Table 2. The K_d for dCTP opposite inosine was 2-fold higher with Y530F(exo$^-$) compared to that of wild-type(exo$^-$) T7 pol, while the K_d for dITP opposite cytosine was the same for both enzymes. This would indicate a need to stabilize the unpaired functional group on the incoming dNTP, and that Tyr530 is in a position to do so in the wild-type(exo$^-$) enzyme. This experiment reveals a more complicated interaction for the E480D(exo$^-$) mutation and is consistent with the role of Glu480 in stabilizing the binding of all four nucleotides. The much weaker binding and slower rate of incorporation for inosine opposite cytosine could be accounted for because the inosine−cytosine base pair may be perturbed slightly relative to a Watson−Crick geometry. The Glu480 residue also appears to be involved in selective stabilization of dTTP and A:T base pairs, and this experiment supports that interpretation as well.

K_d for Dideoxyribonucleotides. Both mutations appear to have had an effect on binding of at least one of the nucleotide substrates. The E480D(exo$^-$) mutant weakened the binding of all four dNTPs, while the Y530F(exo$^-$) mutation had a specific effect on dTTP binding. Analysis of dideoxynucleotide incorporation was conducted to define the role of the 3′-hydroxyl group in the interaction with the E480 and Y530 amino acids in wild-type(exo$^-$) T7 pol.

The maximum rates and equilibrium constants for the ground state binding of dideoxynucleotides (ddNTPs) were determined as described above for the dNTPs. The results for incorporation of ddATP and ddTTP are given in Table 2. For wild-type(exo$^-$), the loss of the 3′-hydroxyl reduced the rate of incorporation 3−6-fold and increased the K_d of ddTTP relative to the K_d for dTTP approximately 2-fold. With Y530F(exo$^-$), the K_d for ddTTP was 4-fold lower than the K_d for dTTP, but the rate of incorporation with this analog was reduced over 45-fold compared to that of dTTP. The K_d for ddATP was increased by 3-fold and the rate of incorporation was reduced by 3-fold relative to dATP with this mutant. Incorporation of ddTTP with E480D(exo$^-$) results in a greater than 100-fold reduction in the rate, but a 2-fold decrease in the K_d for ddTTP relative to dTTP. The K_d for ddATP was increased slightly, with a 5-fold reduction in the rate relative to dATP.

The rate of incorporation of the dideoxynucleotides was reduced with all three enzymes compared to the incorporation rates of the corresponding deoxynucleotides. This is consistent with the 3′-hydroxyl contributing more to the rate of incorporation than to ground state binding.

The increase in binding affinity observed for ddTTP relative to dTTP with both Y530F(exo$^-$) and E480D(exo$^-$) correlates well with the postulate that tighter binding in the ground state reduces the rate of catalysis (Schultz & Schirmer, 1979; Jencks, 1975). For the dideoxy nucleotides, substrate interaction energy is realized as tighter binding rather than being utilized to promote catalysis by transition state stabilization. Since the rate of catalysis with ddTTP is over 100 times slower with E480D(exo$^-$) and 25 times slower with Y530F(exo$^-$), less of the binding energy may be utilized for catalysis contributing to a 3−4-fold higher affinity for these analog with the mutants. Mechanistically this may be understood since both Glu480 and Tyr530 appear to stabilize selectively the binding of dTTP relative to the binding of the other three dNTPs. Because one point of stabilization with the thymidine nucleotide is missing with these mutants, the incorporation of ddTTP may have more flexibility in binding in the active site for ddTTP. The greater flexibility in binding may result in an apparent higher affinity for the nucleotide, but could reduce the rate of incorporation because it is less likely to bind in the correct conformation necessary for the chemical reaction (Patel et al., 1991).

Pyrophosphorolysis. Both mutations appear to have had some effect on the binding and/or stabilization of the correct dNTP. We then examined the effects of the mutations on the kinetics of the reverse of polymerization, pyrophosphorolysis. Pyrophosphorolysis was examined with both mutants and the wild-type(exo$^-$) enzyme using the same stocks of the DNA substrate, dTTP, and pyrophosphate (PP_i). The enzyme was preincubated with 3′-labeled 26/36-mer in the presence of Mg^{2+}, and the reaction was initiated by the addition of PP_i and dTTP. The time course of production of labeled dTTP was determined for each enzyme. The single-exponential rate of dTTP formation for the Y530F(exo$^-$) mutant was unchanged from wild-type(exo$^-$) enzyme, while the rate for the E480D(exo$^-$) mutant was reduced approximately 10-fold (data not shown).

K_d for PP_i: E480D(exo$^-$). Since the Y530F(exo$^-$) mutant did not appear to have a significant effect on the rate of pyrophosphorolyis, the kinetics of this reaction were not pursued further with this mutant. However, for E480D(exo$^-$), the PP_i concentration dependence of the single-turnover rate of pyrophosphorolysis was determined. The maximal velocity approached that observed for wild-type(exo$^-$) while the K_m was increased 2-fold (data not shown).

The slow single-exponential rate of pyrophosphorolysis for the wild-type(exo$^-$) enzyme has been interpreted to mean there is a rate-limiting conformational change step between PP_i binding and the chemical reaction (Patel et al., 1991). The observed single-exponential production of dTTP with both mutants is consistent with the existence of a rate-limiting step preceding chemistry. If the chemical reaction in the forward direction was altered by the mutation directly, it must also be affected in the reverse direction. However, if

conformational changes limit the rate in each direction, different effects of a mutation could be expected in the forward and reverse reactions. In either case, we conclude that the E480 residue primarily interacts with the base structure to stabilize nucleotide binding but does not interact significantly with the pyrophosphate moiety of the nucleotide.

Substitution of Mn^{2+}. T7 DNA polymerase is dependent on Mg^{2+} for polymerization as well as for exonuclease activity. Two residues which serve as Mg^{2+} binding ligands in the exonuclease active site are Glu5 and Asp7 (Patel et al., 1991; Wong, 1990). Polymerization was examined in the presence of Mn^{2+} to test whether Glu480 is involved metal ion binding. The larger ionic radius of Mn^{2+} could possibly counteract the effect of shortening the Glu480 residue by one methylene group in E480D. When dTTP incorporation was measured in the presence of Mn^{2+} for both E480D(exo$^-$) and wild-type(exo$^-$), the effect of substituting Mn^{2+} was a general increase in the rate of polymerization with both enzymes; a specific effect on the E480D(exo$^-$) mutant was not observed (data not shown).

The lack of a specific effect on incorporation catalyzed by the E480D(exo$^-$) mutant with either increasing Mg^{2+} concentration (data not shown) or with substitution of Mn^{2+} suggests that Glu480 does not provide a ligand for metal binding in the active site, or at the very least, does not serve as the primary ligand.

Fidelity of T7 DNA Polymerase. The high fidelity of T7 DNA polymerase is primarily due to the selectivity that occurs in a two-step binding sequence preceding polymerization (Wong et al., 1991). The fidelity of T7 pol without the proofreading exonuclease is approximately 1 error in 10^5-10^6 bases polymerized; thus, these two steps contribute the major portion of the fidelity of this reaction. First, the wrong nucleotides are selected against in the initial binding step with the ground state binding affinity for the wrong nucleotide approximately 250 times lower than for the correct nucleotide. The second point of selectivity occurs at a conformational change preceding the chemical reaction. In the model proposed by Wong et al. (1991), the conformation change following binding of DNA and dNTP acts as a substrate selection gate based largely upon Watson–Crick base pairing geometry (Kati & Johnson, 1994). Initial "loose" binding of the correct substrates induces a conformational change leading to a configuration of the enzyme where the key residues in the active site are brought into proper alignment to provide transition state stabilization for catalysis. Once in this configuration, chemistry occurs very rapidly.

With enzymes containing a 3′–5′ proofreading exonuclease, two reactions govern the fidelity of DNA polymerization: the rate at which an incorrect nucleotide is incorporated into DNA, and the rate at which a correct base is incorporated over a mismatch which determines the selectivity of the exonuclease (Donlin et al., 1991). Since both Y530F(exo$^-$) and E480(exo$^-$) mutants have an effect on the dNTP binding affinity, an analysis of the rates of misincorporation and incorporation past a mismatch are necessary to determine the effect, if any, these mutations have on fidelity of DNA polymerization.

Misincorporation. The single-turnover misincorporation rate for T7 pol has been previously determined to correspond to the rate observed under steady-state conditions (Wong, 1991), so all the misincorporation experiments were done in the steady state with DNA in at least 10-fold excess over the polymerase.

Table 3: Kinetic Constants for Misincorporation

	K_d		
mismatch[a]	wild-type[b] (mM)	Tyr530-Phe (mM)	Glu480-Asp (mM)
G:T	7 ± 1	1.6 ± 0.16	1.4 ± 0.6
T:G	7 ± 1	2.8 ± 0.14	2.7 ± 0.4
C:T	7 ± 1	2.5 ± 0.3	ND[c]
T:C	7 ± 1	2.2 ± 0.12	ND
T:T	7 ± 1	3.2 ± 1.4	ND
C::A:A	0.09 ± 0.1	1.2 ± 0.3	2.2 ± 0.4
	k_{cat}		
mismatch[a]	wild-type[b] (s^{-1})	Tyr530-Phe (s^{-1})	Glu480-Asp (s^{-1})
G:T	0.21	0.13 ± 0.005	0.02 ± 0.003
T:G	0.25	0.11 ± 0.008	0.03 ± 0.001
C:T	0.05	0.03 ± 0.001	<0.001
T:C	0.09	0.13 ± 0.003	<0.001
T:T	0.05	0.03 ± 0.006	<0.001
C::A:A	0.025	0.28 ± 0.1	0.18 ± 0.1

[a] Mismatches are specified by the dNTP:template. For example, G:T refers to a dGTP incorporation with T in the template. C::A:A refers to correct incorporation of dCTP over an A:A mismatch at the primer terminus. [b] Data from Edwards et al. (1994). [c] Not determined.

The formation of a G:T mismatch, the so called wobble mispair (Crick, 1966), proceeds at the fastest rate of mismatches that have been measured with wild-type(exo$^-$) T7 pol (Wong, 1990). The rates of misincorporation of dGTP opposite a template T and dTTP opposite a G were determined for both Y530F(exo$^-$) and E480D(exo$^-$) and compared to the rates for wild-type(exo$^-$) to give the results listed in Table 3.

Mismatches other than G:T occur at slower rates; the formation of a pyrimidine–pyrimidine mismatch is the slowest of all mispairings (Edwards et al., 1994). The misincorporation rates for dCTP and dTTP opposite a template T, and dTTP opposite a template C were determined for the Y530F(exo$^-$) mutant and the results are given in Table 3. Attempts to measure the rates of the same misincorporations with E480D(exo$^-$) were unsuccessful as the rate was too slow to determine under the conditions employed. These rates are less than 0.001 s^{-1}.

The most significant result from this set of experiments is that, unlike wild-type enzyme, binding the wrong nucleotide with both mutants is now saturable exhibiting binding affinities in the range of 1–3 mM. Saturation of the wrong with wild-type(exo$^-$) T7 pol nucleotide could not be observed below 4 mM, and at higher nucleotide concentrations nonspecific inhibition of the reaction occurred. An estimate for the binding affinity for the wrong nucleotide with wild-type(exo$^-$) enzyme is 6–8 mM, based on competition of correct incorporation with the wrong dNTP (Wong et al., 1991).

Overall, both of the mutations show a slight reduction in the fidelity of the reaction. The nucleotide concentration dependence of the misincorporation rate for both mutants and for wild-type(exo$^-$) T7 pol is shown in Figure 5. This demonstrates quite clearly that both mutant enzymes can be saturated with the wrong nucleotide. The selectivity is defined by ratios of k_{cat}/K_m for correct versus incorrect nucleotides (Wong et al., 1991), as summarized in Table 4. The overall reduction in selectivity for correct nucleotides by the Y530F(exo$^-$) mutant has been reduced by 3–7-fold.

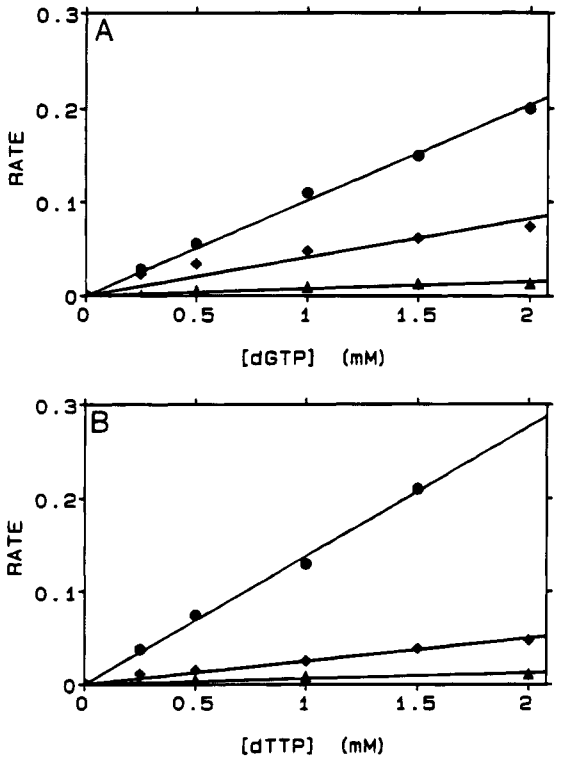

FIGURE 5: Comparison of misincorporation rates. The nucleotide concentration dependence of the first order rate of misincorporation of dGTP opposite T (A) and dTTP opposite G (B) is compared for wild-type(exo$^-$) T7 pol (●), Y530F(exo$^-$) T7 pol (◆), and for E480D(exo$^-$) T7 pol (▲).

Table 4: Comparison of Selectivity against Misincorporation

mismatch	wild-type[a,b]	Try530-Phe	Glu480-Asp
G:T	5×10^5	7.3×10^4	6.3×10^4
T:G	7.4×10^5	2.7×10^5	1.1×10^5
C:T	2.2×10^6	5×10^5	ND[c]
T:C	1.1×10^6	2.0×10^5	ND
T:T	2.2×10^6	6.4×10^5	ND

[a] Selectivity is calculated as the ratio $(k_{cat}/K_m)_{correct}/(k_{cat}/K_m)_{incorrect}$ from the data in Tables 2 and 3. Higher ratios provide greater specificity for correct incorporation. [b] Data from Edwards et al. (1994). [c] Not determined.

The E480D(exo$^-$) mutation has lowered the selectivity by approximately 7−8-fold from that of wild-type(exo$^-$) T7 pol. In both cases, the lower selectivity of the mutant is primarily due to a decreased binding affinity for the correct nucleotide which compensates for the slower rate of misincorporation catalyzed by the mutant.

Correct Incorporation over a Mismatch. We examined next the differences between wild-type(exo$^-$) T7 pol and the two mutant enzymes in the second step in misincorporation, correct polymerization over a mismatch (Table 3). Both mutants shown a much higher maximal rate of incorporation over a mismatch compared to wild-type enzyme, (0.18−0.28 s^{-1} versus 0.025 s^{-1}); however, both mutants also bind the next correct nucleotide much more weakly (1−2 mM versus 87 μM). This effect is comparable to that seen for binding of the correct dNTP with no mismatches in the primer/template.

The rates of incorporation over a mismatch as a function of the nucleotide concentration for both mutants and the wild-type(exo$^-$) T7 pol are shown in Figure 6. This plot demonstrates that at the estimated physiological concentration

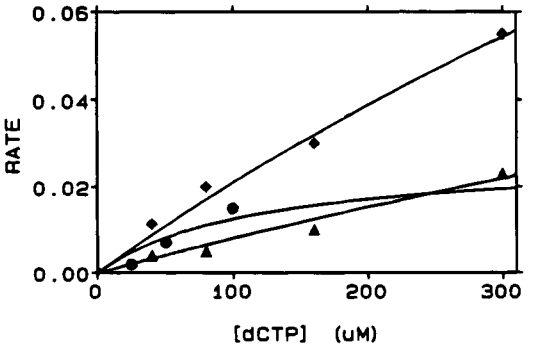

FIGURE 6: Comparison of rates of correct incorporation over a mismatch. The nucleotide dependence of the first order rate of correct incorporation over an A:A mismatch is compared for wild-type(exo$^-$) T7 pol (●), Y530F(exo$^-$) (◆), and E480D(exo$^-$) (▲).

of nucleotides of 100 μM (Neuhard & Nygaard, 1987), Y530F(exo$^-$) has slightly reduced selectivity at this step, while E480D(exo$^-$) has slightly increased selectivity compared to that of wild-type(exo$^-$) T7 pol. Assuming the rate for the transfer from the polymerase to exonuclease site is the same as determined previously (Donlin et al., 1991), the Y530F(exo$^-$) mutant shows a 2-fold reduction in selectivity while E480D(exo$^-$) has increased the selectivity 1.5 times in favor of exonucleolytic removal of the mismatch.

DISCUSSION

Role of Glu480 and Tyr530 in Polymerization. The role of the conserved residue Glu480 is to contribute to the ground state binding of the incoming nucleotides. The increase in K_d for all four dNTPs results from the shifting of the carboxyl group of Glu480 away from the active site and away from the position necessary for it to stabilize nucleotide binding. The higher K_d for dTTP relative to that for the other three nucleotides suggests that this nucleotide may require additional stabilization, provided in part by the carboxylate oxygens of Glu480. Selective stabilization of dTTP may be necessary to compensate for the difference in binding energy of an A:T base pair relative to that of a C:G base pair. Data from incorporation of cytosine opposite inosine support the observation that Glu480 is involved in stabilization of dTTP binding. The results from pyrophosphorolysis and from incorporation of dideoxynucleotide analogs suggest the interaction of Glu480 and the incoming nucleotide occurs with the base and not with the pyrophosphate moiety of the ribose portion of the structure. Glu480 does not appear to be directly involved in binding the Mg^{2+} necessary for catalysis, nor does it appear to be involved in binding DNA. The rate of polymerization approaches that observed for wild-type(exo$^-$) T7 pol if the nucleotide levels are increased to millimolar levels, suggesting that this amino acid does not directly participate in acceleration of chemistry. This is supported by the lack of a significant effect on pyrophosphorolysis when Glu480 is mutated.

Tyr530 also interacts with the incoming nucleotides, and specifically stabilizes the binding of dTTP. A smaller effect on the binding and stabilization of dATP is observed compared to that observed for dTTP. The binding affinity of dCTP and dGTP was not affected by the Y530F mutation. This suggests Tyr530 is involved in the general stabilization of A:T base pairs with a larger effect when dTTP in being incorporated with A in the template. Tyr530's role in stabilizing dTTP binding is supported by the results of the

C:I base pair incorporation. Incorporation of dCTP opposite an inosine mimics the incorporation of dTTP opposite adenine in the sense that there is an unpaired carbonyl oxygen at C-2 of the incoming dNTP; the same trend of a higher K_d for dCTP under these conditions relative to that of wild-type T7 pol is observed. Mutation of Tyr530 does not directly affect the chemistry of polymerization, nor does it affect DNA primer/template binding.

Role of Glu480 and Tyr530 in Fidelity. The results of the investigation into the fidelity of these two T7 pol mutants are consistent with the proposed induced-fit model for fidelity of wild-type T7 pol (Wong et al., 1991). In this model for fidelity, Wong et al., propose that the topology of the substrates recognized by the enzyme is actually an alignment of the substrates to resemble the reaction product. The conformation change preceding chemistry is triggered when the enzyme recognizes this unique topology. If functional groups at the active site have been altered such that the enzyme's ability to recognize this configuration is compromised, then the enzyme may not necessarily recognize a "bad fit" of a substrate in the active site. Removing or shifting a functional group of an active site residue may allow more flexibility in the active site, such that the incorrect dNTP has greater freedom to bind in the active site. However, because of the strict configuration necessary to induce the conformation change preceding chemistry, it would not necessarily increase the rate of misincorporation, but would only increase the affinity for the wrong nucleotide.

The calculations for selectivity of the mutant enzymes compared to those for the wild-type(exo$^-$) T7 pol are listed in Table 4. The selectivity of Y530F(exo$^-$) is reduced only 3−7-fold. The mutant E480D(exo$^-$) has 8-fold reduced selectivity when incorporating a T opposite G because of the much lower affinity for dTTP compared to that of wild-type enzyme which compensates for the slower rate of misincorporation catalyzed by the mutant, relative to the wild type enzyme.

An important question to be addressed is why the mutant polymerases show saturation kinetics with the wrong nucleotide and yet catalyze in the incorporation of mismatches at a slower rate. In order for a wrong nucleotide to be incorporated, the enzyme may still undergo the conformational change preceding chemistry which brings the reactants into the correct conformation for the reaction to take place. Altering the active site by mutating amino acids within the active site may allow tighter binding of the incorrect nucleotide, but the binding energy is not utilized to bring about catalysis, either via the rate-limiting conformational change or by transition state stabilization. With a mismatched nucleotide in the mutated active site, the geometry of the E-DNA−dNTP complex may be even less likely to trigger that conformational change and to initiate chemistry. The higher affinity for the wrong nucleotides can than be explained using the same argument for apparent binding affinity and its relationship to catalysis as was proposed for the observed binding affinity for ddTTP. The tighter binding of the mismatched substrate could suggest that less of the binding energy was utilized for catalysis (Jencks, 1975). The slower rate of misincorporation of the mutants relative to that of wild-type(exo$^-$) is consistent with this interpretation.

Comparison to Other DNA Polymerases. Determination of conserved functional homology between T7 pol and Pol I should be possible since the homologous residues in Pol I (Tyr766 and Glu710) have been mutated and the resulting mutants have been analyzed. In addition, the residue homologous to Tyr530 in ø29 DNA polymerase, Tyr390, has also been mutated, and analyzed, so these results can be compared to a member of Pol-α family of DNA polymerases as well (Blasco et al., 1991). Tyr530 is conserved among all known DNA-dependent DNA polymerases (Delarue et al., 1990).

In Pol I (Klenow), the role of Tyr766 has been defined as binding and stabilization of dNTPs based on work with the Y766S mutant (Polesky et al., 1990). The Y766S mutation increased the K_m for dTTP binding 2−3-fold (Polesky et al., 1990). This mutant was analyzed in a different study to assess its effect on the fidelity of polymerization (Carroll et al., 1991). Y766S had a large effect on fidelity, while the Y766F mutant had a lesser effect. The Y766S mutant had approximately 1.5 times less affinity for dATP (Carroll et al., 1991). From these results Carroll et al. (1991) propose that the phenolic ring of this tyrosine may be important to fidelity because of its ability to exclude water from the active site and thus amplify the binding energy differences between correctly base paired and mispaired nucleotides (Petruska et al., 1986). In ø29 DNA polymerase, a member of the Pol α family of DNA polymerases, the homologous mutation, Y390F, was made and shown to decrease the processivity of polymerization, and so a role in nucleotide binding cannot be ruled out for this tyrosine in this polymerase (Blasco et al., 1991). The ability of this tyrosine to stabilize selectively A:T base pairs and dTTP binding in either Pol I or ø29 DNA polymerase is not known, but cannot be ruled out on the basis of the available data.

Glu480 is conserved among the Pol I family of DNA polymerases, and the homologous residue in Pol I (Glu710) has also been mutated and the product has been analyzed (Polesky et al., 1992). In Pol I this residue is believed to be involved in nucleotide stabilization, as well as in stabilization of DNA. Two mutations of the homologous residue, E710D and E710A, caused a 2−3-fold and 5-fold increase in the K_m for dNTP binding respectively, compared to the 5−40-fold effect observed with E480D in T7 pol. In Pol I, this mutation also caused a greater than 10-fold increase in the K_d for DNA binding, whereas in T7 it caused a slight decrease in the K_d for DNA binding. Polesky et al. (1992) propose the differences observed in DNA and dNTP binding for this mutation are the result of its inability to ligand properly the divalent metal ion necessary for polymerization. However, they did not assess the effect of Mg^{2+} concentration or of any other metal ion on the kinetics of polymerization or on dNTP binding.

While previous work on Pol I indicated an effect on DNA binding, neither mutation examined here altered DNA binding significantly. When an appropriate substrate for T7 pol was being chosen, experiments were conducted with the smaller substrates used in the studies on Pol I (13/20-mer and 9/20-mer; Kuchta et al., 1988). T7 pol did not bind with high affinity to these shorter DNA templates; the longer 25/36-mer proved to be a better substrate. This suggests that a longer segment of DNA is bound in the active site of T7 pol compared to that bound by Pol I, and could explain the lack of significant effect of these mutations on the binding affinity of DNA.

Mutational analyses with both Pol I and ø29 DNA polymerases suggest that the fifth conserved motif in Pol

I-like polymerases (Delarue et al., 1990), and YGDTDS or the Asp−Asp motif in Pol α-like polymerases (Bernad et al., 1990), is directly involved in the chemistry of the polymerization reaction (Polesky et al., 1990, 1992; Blasco et al., 1991). This study on T7 pol mutations E480D and Y530F does not dispute this, as neither mutation appears to be directly involved in chemistry. Rather, the analysis of E480D and Y530F supports the hypothesis that these two conserved residues form part of the nucleotide binding pocket in both T7 pol and in Pol I, and probably in the Pol α-like polymerases as well. Conservation of motifs critical for chemistry and nucleotide binding between the different families of DNA-dependent DNA polymerase would not be unexpected, and fits with the proposal that all DNA polymerases have a common ancestor and have since undergone divergent evolution (Delarue et al., 1990).

This hypothesis is supported by the fact that conservation of a functional motif has previously been shown between the Pol I- and Pol α-like polymerases in the N-terminal portion of the protein containing the 3′ → 5′ exonuclease activity (Bernad et al., 1989). Two conserved amino acids in the 3′ → 5′ exonuclease site have been demonstrated to serve as the ligands for binding metal ion necessary for catalysis, and mutation of these two amino acids results in an exonuclease deficient mutant in Pol I (Derbyshire et al., 1988), in T7 pol (Patel et al., 1991), and in ø29 DNA polymerase (Bernad et al., 1989).

The differences in the physiological roles for Pol I and T7 pol suggest that functional homology will not always be conserved despite the high degree of sequence homology. As was observed in the mechanism of the associated 3′ → 5′ exonuclease activity, the method of transfer of the DNA from site to site on the enzyme was adapted to the processivity constraints on the enzyme (Donlin et al., 1991). Precisely defining the roles of the individual amino acids in the polymerase active site should provide insight into the differences accumulated in these enzymes as they adapted to the different constraints in the host organism.

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