Slow Rate of Phosphodiester Bond Formation Accounts for the Strong Bias that Taq DNA Polymerase Shows against 2′,3′-Dideoxynucleotide Terminators

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ABSTRACT: Taq and T7 DNA polymerases have become basic molecular biology "tools" for DNA sequence analysis. However, Taq, unlike T7 DNA polymerase, is strongly biased against the incorporation of 2′,3′-dideoxynucleotide triphosphates (ddNTPs) indicating very different substrate selectivities. Equilibrium binding and rate constants were measured for 2′,3′-ddNTPs as well as for several other 3′-substituted terminators and compared to 2′-deoxynucleotide substrates (dNTPs). In steady-state experiments, Taq Pol I was strongly biased in favor of dATP[1] over ddATP incorporation by about 700 to 1, in contrast to T7 DNA polymerase which showed a preference of only about 4 to 1. Manganese reduced but did not eliminate selectivity against 2′,3′-ddNTPs. Transient kinetic traces indicated different rate-limiting steps for substrate and terminator incorporation. Further mechanistic studies showed that the binding constants for substrates and terminators were equivalent. However, the rate constants for phosphodiester bond formation for 2′,3′-ddNTPs were 200−3000-fold lower than for dNTPs. Alternative terminators showed only slight improvements. The data were consistent with a model in which both substrates and terminators undergo ground-state binding followed by formation of a tight-binding Enz·DNA·Nucleotide complex. Immediately after complex formation, substrates undergo a rapid nucleoside phosphoryl transfer reaction. However, the reaction rates for terminators were slower presumably due to misalignment of reactive groups in the active site. Thus, the strong bias that Taq DNA polymerase shows against terminators is due to a very slow "chemistry" step. Such a strong bias has several kinetic consequences for DNA sequence patterns. These consequences are discussed in the text.

The thermostable DNA polymerase I, Taq Pol I, from *Thermus aquaticus* has been used extensively in the polymerase chain reaction to amplify small quantities of DNA (Saiki et al., 1988). Due to its high turnover number, lack of a proofreading activity, high-temperature optimum, and ability to incorporate 7-deaza-3-deoxyguanosine efficiently, Taq Pol I has also been used extensively for DNA sequencing (Innis et al., 1988). In particular, it is the ability of this polymerase to amplify small amounts of template DNA through "thermocycling" that has made it an especially useful tool for the high throughput demands of genome sequencing projects, for example. Many different protocols have been developed for thermocycle sequencing both for radiometric (Innis et al., 1988) and for fluorescent detection methods (Prober et al., 1987; Lee et al., 1992). All current protocols, however, rely upon the incorporation of 2′,3′-dideoxynucleotide triphosphates (2′,3′-ddNTPs)[1] in order to terminate chain extension and generate sequence ladders. Although Taq Pol I yields long stretches of DNA sequence, it shows uneven peak height patterns in automated DNA sequence profiles (Lee et al., 1992). Uneven peak heights make automated base calling more difficult and limit the overall readable sequence length. Optimizing reaction conditions and using modified forms of this polymerase have shown substantive improvements; however, peak height profiles for Taq DNA polymerase are less uniform than the profiles for other, noncyclable enzymes, such as T7 DNA polymerase (Fuller, 1992; Rosenthal & Charnock-Jones, 1992).

A comparison of Taq Pol I and T7 DNA polymerases has revealed very different behavior for incorporation of terminators. For example, Tabor and Richardson (1989) showed that when magnesium was the only available divalent cation, Taq Pol I strongly preferred incorporation of dATP over ddATP (≈600 to 1). T7 DNA polymerase, however, exhibited far less preference (only about 4 to 1). Their results further showed that the presence of manganese affected each polymerase differently. T7 DNA polymerase was no longer able to distinguish between the natural substrate and its terminator analog in the presence of manganese (a 1 to 1 incorporation ratio) whereas Taq Pol I still favored dATP over ddATP by a ratio of at least 60 to 1. Divalent cations have also been shown to affect DNA sequence profiles. In the presence of magnesium, profiles generated by T7 DNA polymerase were not uniform. However, in the presence of manganese, peak height profiles were remarkably uniform (Tabor & Richardson, 1989; Fuller, 1992). These observa-

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[1] Abbreviations: dAMP, 2′-deoxyadenine 5′-monophosphate; dATP, 2′-deoxyadenine 5′-triphosphate; ddAMP, 2′,3′-dideoxyadenine 5′-monophosphate; ddATP, 2′,3′-dideoxyadenine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; ddCTP, 2′,3′-dideoxynucleotide 5′-triphosphate; 2′,3′-ddNTP, 2′,3′-dideoxynucleotide 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; 2′-dNTP, 2′-deoxynucleotide 5′-triphosphate; DTT, dithiothreitol; dTTP, 2′-deoxythymidine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; FAM, 5-carboxyfluorescein; β-ME, β-mercaptoethanol; PEI, polyethylimine; PMSF, phenylmethylsulfonyl fluoride; PP_i, inorganic pyrophosphate; SDS, sodium dodecyl sulfate; TRIS, tris(hydroxymethyl)aminomethane.

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tions suggest that there may be a link between substrate versus terminator incorporation ratios and uniformity of peak height profiles in DNA sequence traces.

Uneven peak heights suggest sequence dependent differences in incorporation rates. An understanding of how sequence context might affect these two polymerases differently requires an understanding of the basic kinetic parameters that govern substrate versus terminator incorporation. The main structural difference between 2′-dNTPs ("substrates")[2] and their 2′,3′-ddNTP analogues ("terminators")[2] is that these terminators lack 3′-hydroxyl groups. Despite the fact that the 3′-hydroxyl group on the *incoming* nucleotide is not itself a reactive group, these observations suggested to us that it must be involved in binding substrates and/or in helping to align reactive groups in the active site, thus facilitating phosphodiester bond formation for Taq DNA polymerase but apparently not for T7 DNA polymerase. For example, the 3′-OH group may affect catalysis *directly* through the formation of an essential hydrogen bond with Taq DNA polymerase that stabilizes substrate binding or *indirectly* involved through helping to align the 5′-phosphate group in the active site. In the first case, a missing 3′-OH group would have a dramatic effect on ground-state binding, making terminators bind weaker than substrates. In the latter case, lack of this hydroxyl group would be expected to reduce the rate of terminator phosphodiester bond formation without significantly affecting terminator binding.

Incorporation of naturally occurring 2′-dNTPs versus their 2′,3′-ddNTP is actually a problem of substrate specificity or in the case of a DNA sequencing reaction, *substrate versus terminator selectivity* since both substrates and terminators must compete for the same substrate binding site at each template position during sequencing reactions. The probability of an extension versus a termination event is governed by the ratio of substrate versus terminator selectivity as defined by eq 1 where v is the velocity of terminator ("T" or "dd") or substrate ("S" or "d") incorporation at any given template position, k_{pol} represents the rate of phosphodiester bond formation, and K_d represents the equilibrium binding constant (Fersht, 1985). The first step in understanding the

$$\frac{v_S}{v_T} = \frac{\left[\frac{k_{pol\text{-}d}}{K_d}\right]_S [dNTP]}{\left[\frac{k_{pol\text{-}dd}}{K_{dd}}\right]_T [ddNTP]} \quad (1)$$

mechanism responsible for such disparate behavior between these two DNA polymerases is to measure the kinetic and thermodynamic parameters for substrate versus terminator incorporation or selectivity in order to determine the kinetic basis for the very strong bias that Taq DNA polymerase shows against the incorporation of 2′,3′-ddNTPs.

EXPERIMENTAL PROCEDURES

Enzymes. "KlenTaq," a shortened form of *T. aquaticus* DNA polymerase having an N-terminal deletion of the first 236 amino acids (start sequence: NH_2-MDDLK...), was obtained from an overproducing clone (Barnes, 1992) following induction and purification as described below. This deletion eliminates the entire 5′ to 3′ exonuclease domain of the polymerase. A full-length, exonuclease deficient form of Taq Pol I, AmpliTaq CS, or "G46D," was a gift from D. H. Gelfand (Roche Molecular Systems, Alameda, CA). T7 DNA polymerase was purchased from United States Biochemicals Corporation as "Sequenase 2.0," a formulation consisting of a 3′ to 5′ exonuclease deficient form of this polymerase ("Δ28-G5P"; Fuller, 1992) mixed with an inorganic pyrophosphatase.

Overexpression and Purification of Recombinant KlenTaq DNA Polymerase. To prepare sufficient quantities of KlenTaq polymerase for detailed kinetic analyses, we chose to use an overexpressing recombinant clone encoding KlenTaq that represents an amino-terminal deletion of Taq Pol I as described above (Barnes, 1992). *Escherichia coli* cells carrying a plasmid capable of expressing KlenTaq were grown in Luria broth containing 100 μg of ampicillin/mL at 37 °C to an optical density of 0.5 (A_{550}) and then were induced with the addition of 0.1 mM isopropyl thiogalactoside (IPTG). The induced cells were shaken overnight at 37 °C to maximize expression of KlenTaq and were harvested by centrifugation for 15 min at 7000*g* in a Sorvall GS-3 rotor at 4 °C.

Purification of KlenTaq followed procedures recommended by Barnes (1992) with only slight modifications as outlined below. Harvested cells (27 g wet weight) were resuspended (0.1 g/mL) in lysis buffer (20 mM TRIS-HCl, pH 8.5; 10 mM $MgCl_2$; 16 mM ammonium sulfate; 0.1% NP-40; 0.1% Tween-20; 1 mM EDTA; and 0.7 mM PMSF). Lysozyme (Sigma) was added to a final concentration of 0.2 mg/mL, and the cell suspension was stirred on ice for 1 h and then sonicated. The disrupted cells were placed in a water bath at 80 °C and swirled for 20 min to denature *E. coli* proteins. After the heat step, the lysate was cooled on ice for 20 min and clarified by centrifugation for 15 min at 15 000*g* in a Sorvall SS-34 rotor at 4 °C. The supernatant was decanted and saved.

NaCl (5 M) was added slowly to the supernatant to a final concentration of 0.25 M. DNA was precipitated from the protein solution by dropwise addition of 5% PEI to a final concentration of 0.3%. The cloudy, white suspension was stirred for an additional 25 min at 4 °C and then clarified by centrifugation at 15 000*g* also at 4 °C. The supernatant was decanted and saved.

A Bio-Rex 70 chromatography column (25 × 4 cm) was used to remove the PEI from the protein solution. The column was poured and equilibrated in 0.1 M NaCl in TA buffer (20 mM TRIS-HCl, pH 8.5; 10 mM β-ME; 10% glycerol; 0.1 mM EDTA; 0.1% Triton-X100; and 0.1% Tween-20). The PEI supernatant was diluted with TA buffer to a salt concentration of 0.1 M NaCl, and its conductivity was adjusted to that of the equilibration buffer, after which the supernatant was loaded onto the Bio-Rex 70 column. KlenTaq was washed through the column using equilibration buffer.

To remove contaminating proteins, the Bio-Rex 70 eluate was loaded onto a 10 × 1 cm column of heparin-agarose equilibrated with TA buffer in 0.1 M NaCl. After the column was washed with several column volumes of equilibration buffer, KlenTaq was eluted as a sharp peak by washing the column with 1.0 M NaCl in TA buffer. The heparin-agarose eluate was concentrated to about 25 mL with an Amicon concentrator and then adjusted to 50% glycerol. The protein

[2] We have elected to use the same terminology typically used in DNA sequencing applications.

Table 1: Oligodeoxynucleotides

5′-(FAM)-25/36A1: Single T Incorporation

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FAM-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TAG GTC TTG TTT
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5′-(FAM)-25/36T1: Single A Incorporation

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FAM-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TTG GTC TTG TTT
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5′-(FAM)-25/36C1: Single G Incorporation

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FAM-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TCG GTC TTG TTT
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5′-(FAM)-25/36G1: Single C Incorporation

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FAM-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TGC CTC TTG TTT
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concentrate was dialyzed overnight against storage buffer (20 mM TRIS-HCl, pH 8.5; 100 mM KCl; 2 mM β-ME; 0.1 mM EDTA; 0.1% Triton-X100; 0.1% Tween-20; and 50% glycerol) and stored at −20 °C. The total final yield was determined to be 426 mg of KlenTaq on the basis of the absorbance at 280 using an extinction coefficient of 74 820 M^{-1} cm^{-1} calculated from the projected amino acid composition of KlenTaq.

Analysis of purified KlenTaq using SDS−polyacrylamide gels revealed a major band (≈85% of the absorbance detected on the gel, data not shown) migrating at ≈66 kDa plus a minor band (≈12%) that migrated slightly slower than 66 kDa. Amino acid sequence analysis of the major band showed a sequence start point at amino acid position 273 (or NH_2-LERLEF...) (Lawyer et al., 1989). The slower running, minor band represented the expected amino acid sequence start site for the engineered gene (NH_2-MDDLKL...). These observations showed that, in our hands, most of the polymerase was recovered as a shorter form than expected, beginning at amino acid position 273 rather than at position 236. The additional truncation may be due to proteolysis occurring either *in vivo* during the extended induction period or *in vitro* during the purification steps preceding heat denaturation of the host proteins. We have not pursued these observations further.

Nucleotide Triphosphates and Other Materials. All four dNTPs and all four ddNTPs were purchased as premixed solutions from Pharmacia. [α-^{32}P]ddATP and [8-^{3}H]dATP were purchased from Amersham Corporation. Bulk single-stranded M13mp18 DNA suitable for kinetic experiments was obtained from PanVera Corporation (Madison, WI). Salmon sperm DNA was obtained from Sigma. Alternative terminators, 3′-fluoro-2′,3′-dideoxythymidine triphosphate (3′-F-dTTP), 3′-amino-2′,3′-dideoxythymidine triphosphate (3′-NH_2-dTTP), and 3′-azido-2′,3′-dideoxythymidine triphosphate (3′-N_3-dTTP), were purchased from United States Biochemicals Corporation. Column chromatography materials were purchased from Bio-Rad Laboratories (Richmond, CA).

Synthetic Oligodeoxynucleotides. The synthetic primer and template oligodeoxynucleotides listed in Table 1 were synthesized on an Applied Biosystems 380A DNA synthesizer (DNA Synthesis Facility, Perkin Elmer Applied Biosystems Division) and purified using reverse phase HPLC. Each primer strand was synthesized having a 5′-(FAM)-label (FAM = 5-carboxyfluorescein) using standard chemistries recommended for this instrument. Concentrations of the single-stranded oligodeoxynucleotide fragments were determined by UV absorbance. Duplex primer/template pairs were formed by annealing equimolar amounts of the FAM-labeled 25-mer primer with the appropriate 36-mer template in a solution containing 5 mM TRIS-HCl, 5 mM NaCl, and 0.2 mM EDTA (pH 8.0 at 20 °C) using the following temperature regimen: 5 min at 95 °C; 10 min at 60 °C, and 15 min at room temperature. Duplex DNAs were stored at −20 °C.

Buffers for Rate Measurements. Except where noted in the text, all experiments using either KlenTaq or AmpliTaq CS were carried out at 60 °C in 80 mM TRIS-HCl, 20 mM ammonium sulfate, and 2 mM $MgCl_2$ or 0.6 mM $MnCl_2$ (pH 9.0 at 20 °C. All experiments using T7 DNA polymerase were conducted at 37 °C in 40 mM TRIS-HCl, 1 mM DTT, 50 mM NaCl (pH 7.5 at 20◦ C). To minimize oxidation effects, manganese-containing buffers were prepared fresh daily. All concentrations of reactants are reported as final concentrations after mixing.

Radiometric Assays. The ratio of ddATP to dATP incorporation was based upon a modification of the steady-state competition assay published by Tabor and Richardson (1989). In this case, however, the completed reactions were precipitated, rather than filtered, using salmon sperm carrier DNA according to standard protocols (Maniatis et al., 1982). The actual moles of dAMP and ddAMP incorporated were determined by calculating dpm values through standard quench correction methods and known specific activities of the monomer pools.

Rapid Chemical Quench Experiments. Rapid-quench experiments were carried out using an apparatus designed by Johnson (1986) and purchased from KinTek Corporation (State College, PA). This apparatus has been constructed such that, typically, enzyme plus duplex DNA was loaded into one sample loop (14 μL) and nucleotide−divalent cation combinations were loaded into the other sample loop (14 μL). The reactions were started by rapidly mixing the reactants and then chemically quenching the reaction mixture with 0.36 M EDTA (final concentration; pH 8.0) at varying time intervals ranging from 3 ms to several minutes.

Product Analysis. The 5′-(FAM)-labeled primer/templates were resolved on 16% polyacrylamide/8 M urea sequencing gels and visualized on a Perkin Elmer Applied Biosystems model 373 DNA sequencer using 672 GeneScan software for peak identification and relative fluorescence measurements. The amount of 26-mer product produced as a function of time was calculated by normalizing the product peak areas for each time point. The resulting curves were fitted to biphasic, single-exponential, or rectangular hyperbola equations depending upon the experiment (as described in the text) by nonlinear regression analysis using curve-fitting software purchased from Synergy Software (Reading, PA).

Measuring Rates of Phosphodiester Bond Formation and Equilibrium Binding Constants. The nucleotide concentration dependence of the rate of 26-mer product formation was measured using the rapid chemical quench apparatus under polymerase excess (500 nM) over DNA (100 nM) reaction conditions. Reaction times for each nucleotide concentration varied depending upon the type of nucleotide being tested, but typical times ranged from 3 ms to 1 s for substrates and

2 to 60 s for terminators as described in the text. Plots of product formation rates versus nucleotide concentrations yielded typical rectangular hyperbolas from which the maximum rate of phosphodiester bond formation, k_{pol}, and the equilibrium binding concentration, K_d, could be calculated as described in the text.

Measurement of the Polymerase Release Rate. The dissociation rate of polymerases from the 5′-(FAM)-25/36-mer primer/template was measured using a modification of the pre-steady-state setup on the rapid chemical quench flow apparatus [see also Patel et al. (1991)]. An appropriate 5′-(FAM)-25/36-mer (200 nM) was preincubated with polymerase (100 nM) in the absence of either substrate or magnesium. An unlabeled 41/54-mer (30 μM) ("*TRAP DNA*") was then added to the above complex and incubated for different time intervals ranging from 5 to 500 ms. At the end of the incubation period, magnesium (2 mM) plus only the next correct deoxynucleotide (400 μM) was added and the reaction was allowed to proceed for six times the measured half-life for a single turnover of the appropriate base (typically 40−70 ms). The reaction was stopped with EDTA (final concentration 360 mM). The amount of 26-mer product was measured as before on 16% gels, but in this case, the product represented the amount of polymerase still bound to the enzyme·DNA complex and still able to react to form a detectable product. The data followed single-exponential decay curves. The rate calculated from these curves represented the polymerase release rate or k_{off}.

RESULTS

The DNA-dependent DNA polymerase, Taq Pol I, from *T. aquaticus* strain YT1 has been cloned and overexpressed in *E. coli* (Lawyer et al., 1989; Engelke et al., 1990). The full-length form of this polymerase consists of a single polypeptide chain of 832 amino acids having a calculated molecular weight of 93 910. Two catalytic domains showing high amino acid sequence homology to *E. coli* Pol I have been identified: a 5′ to 3′ exonuclease domain in the first third of the molecule and a DNA synthesis domain in the C-terminal two-thirds. Unlike *E. coli* Pol I, however, no proofreading or 3′ to 5′ exonuclease activity has been detected nor is there any region in the molecule that shows sequence homology to the proofreading domains in other Pol I type polymerases (Tindall & Kunkel, 1988). "Engineered" forms of Taq Pol I have become increasingly popular "tools" in molecular biology for amplifying small quantities of template DNA into readable sequence ladders. One such form, for example, has been designated AmpliTaq CS, a full-length version that has been rendered exonuclease deficient by a point mutation substituting aspartic acid for glycine at position 46 ("G46D"; D. Gelfand, personal communication). Another engineered form, "KlenTaq", represents an N-terminal truncation eliminating the entire 5′ to 3′ exonuclease region and leaving only the polymerase domain (Barnes, 1992), making it analogous to the Klenow fragment of *E. coli* DNA polymerase, "*E. coli* Pol I KF." We chose to examine these two forms of Taq Pol I and present experiments that show that the very strong bias that Taq Pol I has against 2′,3′-ddNTP terminators is due to much lower rate constants for terminator phosphodiester bond formation rather than to differences in binding of terminators versus naturally occurring 2′-dNTP substrates. Such a strong bias against terminators has several kinetic consequences which will be discussed further in terms of DNA sequence profiles.

Table 2: Relative Incorporation of dAMP and ddAMP by KlenTaq and T7 DNA Polymerases in the Presence of Mg^{2+} and Mn^{2+}

	dAMP/ddAMP incorporation ratio[a]	
	KlenTaq DNA polymerase[b]	T7 DNA polymerase[c]
5.0 mM Mg^{2+}	670	3.2
10.0 mM Mg^{2+}	700	2.7
2.0 mM Mn^{2+}	16	1.2
5.0 mM Mg^{2+} and 2 mM Mn^{2+}	17	1.0
10.0 mM Mg^{2+} and 2 mM Mn^{2+}	17	1.0

[a] The ability of KlenTaq DNA polymerase to incorporate dAMP and ddAMP was measured using primed M13mp18 DNA. Reaction mixtures contained 8 nM primer/template DNA complex, 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, and 100 μM [^{3}H]dATP (500 dpm/pmol) plus 10 μM [α-^{32}P]ddATP (5000 dpm/pmol) in 80 mM TRIS·HCl, 20 mM ammonium sulfate (pH 9.0 at 20 °C) with the indicated concentrations and mixtures of divalent cations. Reactions were incubated at 60 °C. [b] The ability of T7 DNA polymerase to incorporate dAMP and ddAMP was also measured using primed M13mp18 DNA. Reaction mixtures contained 20 nM primer/template DNA complex, 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, and 100 μM [^{3}H]dATP (500 dpm/pmol) plus 10 μM [α-^{32}P]ddATP (5000 dpm/pmol) in 20 mM TRIS-HCl, 5 mM DTT (pH 7.5 at 20 °C) with the indicated concentrations and mixtures of divalent cations. Reactions were incubated at 37 °C. [c] Reactions were started by the addition of 10 pmol of the enzyme, further incubated for 10 min, and then stopped on ice, and samples were precipitated using sonicated carrier salmon sperm DNA and 1 M HCl according to standard procedures (Maniatis et al., 1982). The actual moles of dAMP and ddAMP incorporated were determined from measuring the radioactivity in the precipitated samples and converting it to dpm via standard quench correction curves. The data presented above represent the ratio of the moles of incorporation of substrate dAMP/moles of terminator ddAMP.

Processive Steady-State Substrate/Terminator Incorporation Ratios. Certain divalent cations have been shown to affect peak height profiles in DNA sequence traces (Tabor & Richardson, 1989; Fuller, 1992). The effect of different divalent cations on ddATP versus dATP incorporation by Taq Pol I was investigated using a modified radiometric method similar to the steady-state competition assay published by Tabor and Richardson (1989). These assays were based upon processive incorporation of all four deoxynucleotides by KlenTaq using M13mp18 as a template with a standard sequencing primer, "(−47)24-mer" (Messing et al., 1984). Terminator versus substrate incorporation ratios were calculated from predetermined specific activities of the [α-^{32}P]ddATP/[8-^{3}H]dATP monomer pool.

The optimal magnesium concentration for maximum KlenTaq activity in 80 mM TRIS-HCl plus 20 mM ammonium sulfate (pH 9.0) was broad, ranging from 2 to 5 mM (data not shown) which was the same concentration range for the lowest substrate to terminator ratios. KlenTaq showed a strong preference for dATP over ddATP incorporation ranging from ≈800:1 to 700:1 as shown in Table 2. Substrate to terminator incorporation ratios for T7 DNA polymerase were lower at all magnesium ion concentrations tested and averaged only about 3 to 1, suggesting that under typical DNA sequencing reaction conditions, the bacteriophage polymerase is less discriminating against 2′,3′-ddNTPs compared to KlenTaq.

Manganese has been shown to profoundly affect DNA polymerase activity (Beckman et al., 1985). For example, Tabor and Richardson (1989) published that manganese

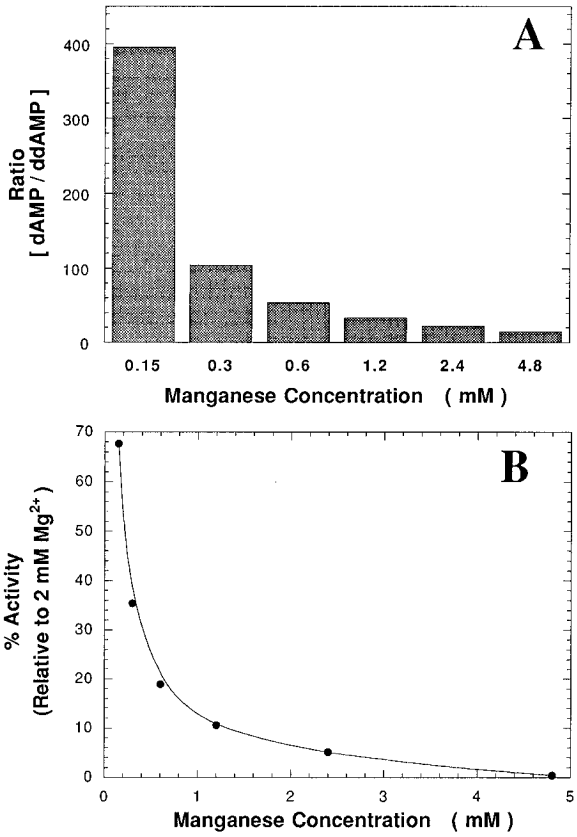

FIGURE 1: Manganese effects on KlenTaq. (A) The bar plot shows the dAMP/ddAMP incorporation ratio for KlenTaq in the presence of increasing concentrations of manganese. Reaction components and samples were handled in the same manner as described for Table 2. The moles of dAMP and ddAMP incorporated were calculated according to the method described under Table 2 and in Experimental Procedures. (B) The line plot (●) shows KlenTaq activity at increasing concentrations of manganese alone relative to the level of polymerase activity observed for 2 mM magnesium alone.

causes T7 DNA polymerase to incorporate dATP and ddATP equally well even when magnesium was also present in the same reaction mixture. They further showed that manganese caused T7 DNA polymerase to yield uniform peak heights in DNA sequencing traces, suggesting that when this polymerase can no longer distinguish between substrate and terminator structures, terminator incorporation from one to another template position was uniform. They reported that Taq Pol I, on the other hand, still strongly preferred dATP over ddATP (about 60 to 1) even in the presence of manganese, but no information was published concerning any effects on sequence profiles. Therefore, we chose to further investigate the effects of manganese as well as other divalent cations on Taq DNA polymerase performance.

Figure 1A shows that as the concentration of manganese was increased in the reaction mixtures, the substrate to terminator incorporation ratios decreased for KlenTaq. However, at all manganese concentrations tested, KlenTaq activity was severely inhibited relative to its activity in 2 mM magnesium, alone, as shown in Figure 1B. Consequently, it was not possible to reduce the preference shown by KlenTaq for substrates over terminators below about 17 to 1, as shown in Table 2. T7 DNA polymerase, on the other hand, was only slightly inhibited by manganese (≈80%−85% of the values observed with 2 mM magnesium, alone), which was constant over the concentration range 0.3−4.8 mM. T7 DNA polymerase also showed a constant incorporation ratio of ≈1 whenever manganese was present at a concentration greater than 30 μM (data not shown).

Other divalent cations tested for their effects on KlenTaq performance were cobalt(II), nickel(II), copper(II), and zinc. Only cobalt and zinc supported KlenTaq activity. Of all of the divalent cations tested, only manganese caused a differential incorporation effect, i.e., lower dATP coupled with significantly higher ddATP incorporation. Manganese also behaved as a "dominant" cation over cobalt(II) and zinc even when magnesium was present as well in the reaction mixtures (data not shown).

DNA sequencing traces revealed that while manganese appeared to qualitatively improve the uniformity of the peak height profiles, the patterns were still far less uniform than those shown by T7 DNA polymerase in the presence of magnesium alone (data not shown).

Pre-Steady-State, Single-Turnover Incorporation Rates. Although informative, steady-state assays could not reveal mechanistic details concerning substrate versus terminator binding or rates of phosphodiester bond formation. To further analyze incorporation preferences, we chose to measure pre-steady-state, single-nucleotide incorporation kinetics. The aim in this approach was to be able to observe individual kinetic steps along the forward polymerization pathways of substrates and terminators using synthetic oligodeoxynucleotide primer/template pairs as the duplex DNA. Care was taken to minimize the differences in the sequences of the individual fragments in order to minimize any sequence context effects. For the primer/template sequences listed in Table 1, it was possible to derive a single set of rate and binding constants that were internally consistent under the reaction conditions explored.

Burst Kinetics. Because of the very fast incorporation rates of dNTPs shown by KlenTaq and especially by T7 DNA polymerase, it was necessary to use a rapid chemical quench flow apparatus (Johnson, 1986). Initial experiments were conducted under DNA excess conditions in order to observe the first and subsequent turnovers of the enzyme. In the case of Taq DNA polymerase, however, the truncated form (KlenTaq) bound the primer/templates shown in Table 1 too weakly to preform an E·DNA complex ($K_{DNA} \geq 160$ nM) compared to the full-length form of this polymerase ($K_{DNA} \approx 10$ nM; data not shown). Therefore, the full-length, exo$^-$ form, AmpliTaq CS, was used to examine the burst kinetic characteristics of this polymerase. The reactions were carried out by mixing a preincubated solution containing AmpliTaq CS (100 nM; all concentrations are listed as final concentrations after mixing in the instrument) plus 5′-(FAM)-25/36A (500 nM) with a solution containing Mg^{2+} (2 mM) plus dTTP (400 μM) in the rapid chemical quench instrument. Polymerization was stopped with EDTA (final concentration 0.36 M) at time intervals ranging from two to several hundred milliseconds. The resulting time course for dTTP incorporation as shown in Figure 2A exhibits a burst kinetic pattern. The first, exponential phase represents the first turnover of the polymerase, with a rate of ≈50 s^{-1} and a burst amplitude of ≈100 nM. Subsequent enzyme turnovers occur at a much slower rate of ≈3 s^{-1}. Under these reaction conditions, a burst pattern is typical for DNA polymerases. The first phase represents the rapid forward polymerization caused by mixing a preformed E·DNA complex with substrate plus divalent cation. The second phase represents the rate of additional

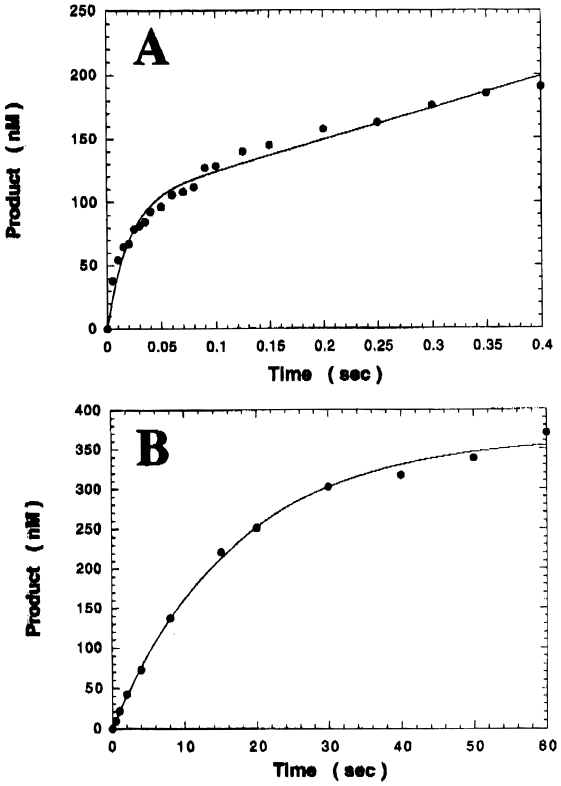

FIGURE 2: Pre-steady-state kinetics of dTTP and ddTTP incorporation. (A) A preincubated solution of AmpliTaq CS polymerase (100 nM) plus 5′-(FAM)-25/36A primer template DNA (500 nM) was mixed with Mg^{2+} (2 mM) plus dTTP (400 μM) in a rapid quench flow instrument (all concentrations are final concentrations after mixing in the instrument). The reactions proceeded for 3−500 ms and then were quenched by the rapid addition of EDTA to a final concentration of 360 mM. Samples (typically 4 fmol of total primer/template DNA per lane) were loaded onto a 16% polyacrylamide/8 M urea gel, and fluorescent signals in the 25-mer primer and 26-mer product bands were measured using a model 373 DNA Sequencer fitted with GeneScan Fragment Analysis software. The amount of fluorescent signal in the product bands was normalized as described in Experimental Procedures. The plot (●) shows a fit to a burst equation [$k_{obs} = A(1 - \exp^{-rt}) + k_{ss}t$] where k_{obs} is the observed rate of the reaction, A is the burst amplitude which is proportional to the active site or polymerase concentration, r is the burst rate, t is time, and k_{ss} is the steady-state turnover rate. Under these reaction conditions, the burst amplitude was 100 ± 5 nM with a burst rate equal to 53 ± 7 s^{-1} and a steady-state rate of 2.5 ± 0.2 s^{-1}. The second or slower phase was equivalent to the steady-state, single-nucleotide dTMP incorporation rate. (B) A preincubated solution of AmpliTaq CS polymerase (100 nM) plus 5′-(FAM)-25/36A primer template DNA (500 nM) was mixed with Mg^{2+} (2 mM) plus ddTTP (400 μM) in a rapid quench flow instrument (all concentrations are final concentrations after mixing in the instrument). The reactions proceeded for 2−60 s. Reactions were then quenched by adding EDTA to 360 mM, and the data were treated in the same manner as described for Figure 2A. The plot shows an initial rate (●) of ≈0.2 s^{-1}.

incorporation events requiring dissociation of the polymerase from the E·DNA complex. A burst pattern indicates that the rate of polymerization was faster than the rate of polymerase release. Therefore, the rate-limiting step during single-nucleotide incorporation for Taq DNA polymerase must occur after the nucleoside phosphoryl transfer reaction or "chemical" step in its polymerization pathway (Johnson, 1993). Independent measurements of the enzyme-release rate using an "enzyme-trap" experiment (as described in Materials and Methods) confirmed that the slow second phase in these burst patterns was, indeed, caused by the slow dissociation of the polymerase from the E·DNA complex ($k_{off} = 2.4 \pm 0.2$ s^{-1}).

Pre-steady-state experiments for measuring Mg^{2+}·ddNMP incorporation by AmpliTaq CS were conducted under the same reaction conditions described above, except that much longer reaction times were necessary. There was a notable difference in the incorporation curves for terminators compared to substrates. As shown in Figure 2B, ddTMP incorporation follows a smooth curve with an initial rate of ≈0.2 s^{-1} rather than burst kinetics. The absence of a pre-steady-state burst for ddTMP incorporation clearly indicates that the rate-limiting step for terminator incorporation must occur at either the "chemistry" step or at a step before the chemistry step (Johnson, 1993). Replacing magnesium with manganese did not restore burst kinetics for ddTMP incorporation (data not shown).

The pre-steady-state incorporation patterns for T7 DNA polymerase for both dTTP and ddTTP showed a burst of incorporation for magnesium (alone) and for manganese (alone) indicating that the rate-limiting step occurs after the chemistry step for this enzyme for both substrates and terminators [data not shown; see also Patel et al. (1991)]. These results supported our earlier steady-state results showing that a missing 3′-OH group on 2′,3′-dideoxynucleotide terminators profoundly affects the ability of Taq, but not T7 DNA polymerase, to incorporate this nucleotide analog. For Taq DNA polymerase, the 3′-OH group on the "incoming" nucleotide appears to affect reactivity even though it is not a reactive group.

Binding and Rate Constant Measurements. The K_d or equilibrium dissociation constant for dNTP incorporation was determined by measuring the rate of conversion of the Enz·-25/36-mer complex to an Enz·26/36-mer using a fixed concentration of Enz·DNA (500 nM enzyme plus 100 nM appropriate primer/template DNA) and increasing concentrations of the appropriate nucleotide. As shown in Figure 3A for KlenTaq, the rate of Enz·26/36A formation increased with increasing dTTP concentration over the range 2−200 μM. When these rate data were plotted versus nucleotide concentration as shown in Figure 3B, the data satisfied the pathway shown in eq 2,

$$E{\cdot}D_n + dNTP \overset{K_d}{\rightleftharpoons} E{\cdot}D_n{\cdot}dNTP \overset{k_{pol}}{\rightleftharpoons} E{\cdot}D_{n+1}{\cdot}PP_i \quad (2)$$

$$k_{obs} = \frac{k_{pol}[dNTP]}{[dNTP] + K_d}$$

where k_{obs} is the observed rate of the reaction, k_{pol} is the maximum rate of phosphodiester bond formation, and K_d represents the equilibrium dissociation constant or binding for the nucleotide. Measurements for each of the four naturally occurring dNTPs are listed in Table 3. The values of $k_{pol\text{-}d}$ for KlenTaq in the presence of 2 mM magnesium were determined to be (in s^{-1}) 31 for dTTP, 38 for dATP, 52 for dGTP, and 21 for dCTP. The corresponding K_d values in the presence of 2 mM Mg^{2+} were determined to be (in μM) 57 for dTTP, 52 for dATP, 36 for dGTP, and 35 for dCTP.

Measurement of K_{dd} for ddNTP Incorporation. The K_{dd} values for each of the 2′,3′-dideoxynucleotide terminators used in typical DNA sequencing protocols were measured in the same manner as for dNTPs except that longer time

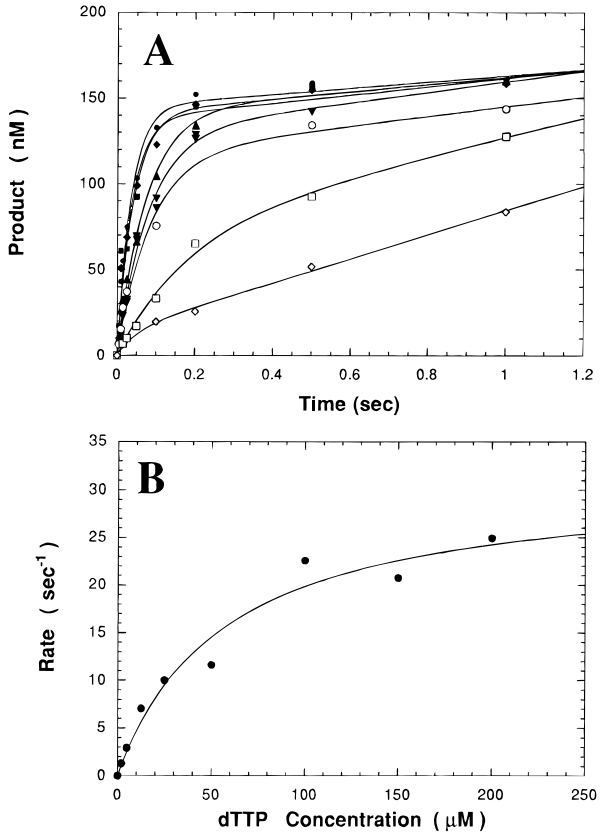

FIGURE 3: Pre-steady-state burst rate dependence on Mg^{2+}·dTTP concentration. (A) A preincubated solution of KlenTaq (500 nM) plus 5′-(FAM)-25/36A (100 nM) was mixed with increasing concentrations of dTTP plus Mg^{2+} (2 mM) in a rapid chemical quench flow instrument (all concentrations are final concentrations after mixing in the instrument). Reactions were allowed to proceed for various time intervals, and samples were processed as described as in the legend to Figure 2A. The dTTP concentrations were (●) 200 μM; (■) 150 μM; (◆) 100 μM; (▲) 50 μM; (▼) 25 μM; (○) 12.5 μM; (□) 5 μM; and (◇) 2 μM. The solid lines represent best fits to the data. (B) The pre-steady-state rates obtained from the data shown above were plotted against dTTP concentrations. The rate data (●) were fitted to a hyperbola, as described in the text, which gave the Mg^{2+}·dTTP K_d of 57 ± 16 μM and a maximum incorporation rate of 31 ± 3 s^{-1}.

courses were necessary to compensate for much slower incorporation rates. Figure 4A shows a series of time courses for the incorporation of Mg^{2+}·ddTMP. Figure 4B represents a plot of the rates of incorporation versus terminator concentrations. Each of the other ddNTPs exhibited similar behavior. As listed in Table 3, dideoxynucleotide terminators show binding values comparable to their deoxynucleotide counterparts, except for ddATP which binds about 5 times weaker than the other ddNTPs. However, the maximum rates of phosphodiester bond formation for ddNTPs were much slower than their natural counterparts, ranging from 0.23 s^{-1} for ddGTP (≈200-fold slower than dGTP) to 0.011 s^{-1} for ddTTP (≈3000-fold slower than dTTP). These observations indicate that the rate-limiting kinetic parameter for ddNTP incorporation was not the binding of these analogs to the Enz·DNA complex but rather their rates of phosphodiester bond formation.

Manganese Effects on Terminator Binding and Polymerization Rates. Table 3 shows manganese effects on the rate and binding constants for substrates and terminators for both polymerases. In the case of dTMP incorporation by KlenTaq, the presence of manganese increased the rate of

Table 3: Rate and Binding Constants for 2′-Deoxy- and 2′,3′-Dideoxynucleotides

	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})
	2 mM Mg^{2+}		
KlenTaq			
dTTP	31 ± 3	57 ± 20	6×10^5
dATP	38 ± 2	52 ± 7	7×10^5
dGTP	52 ± 1	36 ± 3	1×10^5
dCTP	21 ± 4	35 ± 2	6×10^5
ddTTP	0.011 ± 0.0005	66 ± 8	170
ddATP	0.024 ± 0.002	270 ± 50	90
ddGTP	0.23 ± 0.022	48 ± 9	4800
ddCTP	0.030 ± 0.003	58 ± 10	500
	0.6 mM Mn^{2+}		
dTTP	190 ± 10	27 ± 5	7×10^6
ddTTP	2 ± 0.07	28 ± 3	8×10^4
	10 mM Mg^{2+}		
T7 DNA polymerase			
dTTP	137 ± 10	13 ± 3	1×10^7
ddTTP	24 ± 2	45 ± 9	6×10^5
	10 mM Mn^{2+}		
dTTP	235 ± 20	12 ± 3	2×10^7
dTTP	140 ± 8.0	14 ± 3	1×10^7

phosphodiester bond formation by about 6-fold (190 s^{-1} for 0.6 mM Mn^{2+} compared to 31 s^{-1} for Mg^{2+}) while also increasing its binding by approximately 2-fold (27 μM over 57 μM). Manganese had an even greater effect on ddTMP incorporation. The rate of terminator phosphodiester bond formation was increased 190-fold (2.1 s^{-1} compared to 0.011 s^{-1} in 2 mM Mg^{2+}) which was also coupled to 2-fold tighter binding (28 μM over 66 μM).

The effects of manganese on the rate constants described above appear to contradict the results shown in Figure 1B which would predict inhibition of incorporation. However, in burst kinetic experiments, we have found that manganese increases burst rates for dTMP incorporation while reducing the rate of subsequent turnovers (data not shown). We interpret this to mean that manganese increases the rate of the first incorporation event but inhibits further incorporation events. Thus, measurements based upon single-nucleotide incorporation show higher rates of incorporation and higher rate constants than the same measurements made in the presence of magnesium; however, measurements relying on multiple incorporation events such as steady-state experiments show overall inhibition.

Compared to the results for KlenTaq, the presence of manganese had a lesser effect on rate and binding constants measured for T7 DNA polymerase. For the natural substrate, dTTP, manganese increased the rate of phosphodiester bond formation by only about 2-fold (235 s^{-1} over 137 s^{-1}) without showing any effect on substrate binding (12 μM compared to 13 μM). For the terminator, ddTTP, manganese showed a modest increase in bond formation rate of about 6-fold (140 s^{-1} over 24 s^{-1}) coupled with about 3-fold tighter binding.

The apparent second-order selectivity constant, k_{pol}/K_d, is actually a measure of how well an enzyme would incorporate two different types of molecules that compete for the same substrate binding site when present in the same reaction mixture (Fersht, 1985). Table 3 shows that KlenTaq would be expected to strongly incorporate substrates over termina-

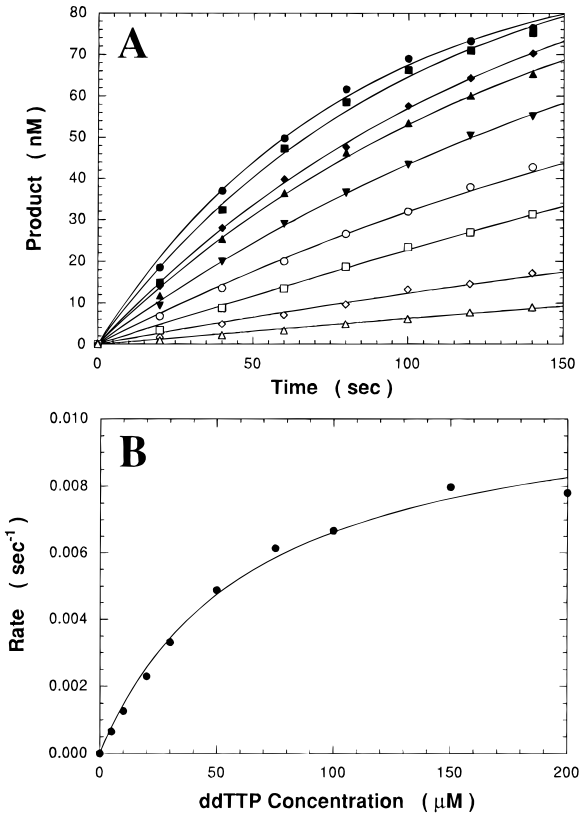

FIGURE 4: ddTTP concentration dependence on the pre-steady-state burst rate. (A) A preincubated solution of KlenTaq (500 nM) plus 5′-(FAM)-25/36A (100 nM DNA) was mixed with increasing concentrations of ddTTP plus Mg^{2+} (2 mM) in a rapid quench flow instrument. Reactions proceeded for various time intervals as indicated and were stopped using EDTA as described above. The ddTTP concentrations were (●) 200 μM; (■) 150 μM; (◆) 100 μM; (▲) 75 μM; (▼) 50 μM; (○) 30 μM; (□) 20 μM; (◇) 10 μM; and (△) 5 μM. The solid lines represent single-exponential fits to the data. (B) The pre-steady-state rates obtained by fitting the data shown above were plotted against ddTTP concentrations. The rate data (●) were fitted as described in the text, which gave the Mg^{2+}•ddTMP K_{dd} of 66 ± 8 μM and a rate constant of 0.011 ± 0.0005 s^{-1}.

tors in the presence of magnesium (≈6×10^5 M^{-1} s^{-1} over ≈200 M^{-1} s^{-1} or ≈3000 to 1 preference for dTTP, for example) and that manganese reduced this bias (7×10^6 M^{-1} s^{-1} over 8×10^4 M^{-1} s^{-1} or ≈90-fold preference). In the presence of magnesium, T7 DNA polymerase would be expected to show far less preference for dTTP over ddTTP (1×10^7 M^{-1} s^{-1} compared to 6×10^5 M^{-1} s^{-1} or only ≈20-fold), and manganese appeared to virtually eliminate this much lower preference (2×10^7 M^{-1} s^{-1} over 1×10^7 M^{-1} s^{-1}, or only about 2 to 1).

Rate and binding constant data indicated that KlenTaq binds both substrates and terminators to the same extent (except for ddATP) and that manganese showed a far greater effect on k_{pol}. Taken together, these observations suggested that manganese may exert its effect through helping to align the reactive groups in the active site of this polymerase and that the presence of a 3′-substituent on the furanose ring of substrates may exert a similar effect. Conversely, the absence of a 3′-OH group on 2′,3′-dideoxynucleotides may result in a ring conformation that causes misalignment of their reactive groups in the active site of Taq DNA polymerase.

Measurement of K_{dd} for Alternative Terminators. 2′,3′-Dideoxynucleotides are used as DNA sequencing reagents because, once incorporated, they do not have an extendible 3′-hydroxyl group. In fact, 2′,3′-ddNTPs are the only type of terminator currently used in enzyme-based DNA sequencing. Alternative molecules with nonextendible 3′-substituents have been described in the literature, however. For example, 3′-fluoro-2′,3′-dideoxynucleotides, 3′-F-dNTPs (Chidgeavadze et al., 1985), and 3′-amino-2′,3′-dideoxynucleotides, 3′-NH_2-dNTPs (Chidgeavadze et al., 1984), can generate readable sequence ladders under appropriate reaction conditions. Furthermore, 3′-F-dNTPs have been shown to be effective chain terminators at one-tenth the concentration typically used for 2′,3′-dNTPs (Chidgeavadze et al., 1985). We were interested, therefore, to measure basic rate and binding constants for some of these alternative terminators to determine what effect, if any, these alternative 3′-substituents might have on polymerase performance.

Pre-steady-state incorporation experiments under polymerase excess conditions were performed in the same manner as described above for 2′,3′-ddNTPs, and the results are summarized in Table 4. In the presence of magnesium, the k_{pol} values for these alternative terminators were similar (about a 2-fold range). The K_d values indicated somewhat tighter binding than 2′,3′-ddTTP, however, ranging from about 2-fold tighter binding for 3′-N_3-dTTP to about 7-fold tighter binding for 3′-(NH_2)-dTTP. It is curious that one of these alternative terminators which lacks any 3′-substituent at all, namely, d4T-TP, also showed about 3-fold tighter binding, suggesting that 3′-substituents may not be directly involved in binding to the enzyme but rather in some other mechanism, such as alignment of reactive groups in the active site.

Our results for KlenTaq were consistent with those published for *E. coli* Pol I (Chidgeavadze et al., 1984). In the presence of magnesium, 3′-F-dTTP showed a selectivity ratio approximately 7-fold higher than ddTTP which predicts that a 7-fold lower concentration of this alternative terminator should give comparable sequencing results. Actual DNA sequence profiles based upon "titrating" a constant concentration of dTTP with decreasing concentrations of 3′-F-dTTP indicated that it was possible to generate readable sequence ladders comparable to those observed using 2′,3′-ddTTP but with only about one-seventh to one-tenth as much of this alternative terminator. Furthermore, side-by-side comparisons showed that the peak height patterns for 3′-F-dTTP were somewhat more uniform than those generated by 2′,3′-ddTTP (data not shown).

While not intended to be an exhaustive study of the effects of alternative 3′-substituents, these results were instructive. Except for 3′-N_3-TTP which may be affected by size and 2′,3′-didehydro-TTP (d4T-TP) which contains a "distorted" furanose ring resulting from the double bond, these alternative terminators can be roughly ranked in terms of their selectivity constants and the electronegativity of their 3′-substituents (Guschbauer & Jankowski, 1980): F > NH_2 > (d4T or "minus H") > N_3 > H.

Enzyme•DNA•Nucleotide Dissociation Rate Measurements. Unlike dNTPs, 2′,3′-ddNTP terminator incorporation profiles did not follow burst kinetics (Figure 2B), indicating that the rate-limiting step for their incorporation must occur at or before the nucleoside phosphoryl transfer step. Pre-steady-state nucleotide titration experiments showed that binding of substrates and terminators was equivalent; however, the rate of phosphodiester bond formation by terminators was

Table 4: Comparison of Rate and Binding Constants for Alternative Terminators for KlenTaq

	2 mM Mg^{2+}			0.6 mM Mn^{2+}			
	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})	k_{pol} (s^{-1})	K_{dd} (μM)	k_{pol}/K_{dd} (M^{-1} s^{-1})	Mn/Mg
dTTP	31	57	6×10^5	190	28	7×10^6	10×
ddTTP	0.011	66	170	2.0	28	8×10^4	400×
3′-N_3-dTTP	0.012	32	380	ND	ND		
d4T-TP	0.008	18	450	4	17	2×10^5	500×
3′-(NH_2)-dTTP	0.007	8	900	7	13	5×10^5	600×
3′-F-dTTP	0.016	13	1200	6	16	4×10^5	300×

200−3000-fold slower than substrates. Therefore, a missing 3′-OH group does not affect ground-state terminator binding but must somehow either reduce the rate of the chemical step, itself, or another step immediately before the chemistry step through some mechanism other than direct binding. Two plausible mechanisms are that the 3′-OH group affects the rate of a putative conformational change in the polymerase which occurs after nucleotide binding and immediately before chemistry or that the 3′-OH group may exert its effect through influencing the conformation of the furanose ring which in turn affects the alignment of reactive groups in the active site or both. By measuring the dissociation rate of DNA from the E·DNA complex with or without the next, correct nucleotide present in the reaction mixture under conditions where the chemistry step is blocked, it was possible to infer whether or not there may be another step between nucleotide binding and the nucleoside phosphoryl transfer reaction (Kati et al., 1992). An additional step involving a conformational change in HIV RTase manifests itself as an inhibition of the rate of single ddNTP turnover caused by the formation of a tighter binding complex (R. Goody, personal communication). Thus, the steady-state turnover rate for the ternary E·DNA_{dd}·dNTP complex is slower than the turnover rate of the binary E·DNA_{dd}. In the case of AmpliTaq CS, this experiment consisted of measuring the steady-state turnover rate of ddTTP incorporation in the absence or presence of dCTP or of ddCTP to determine (1) if the formation of a tighter binding complex occurs in the polymerization pathway catalyzed by Taq DNA polymerase and (2) if the absence of a 3′-OH group has any influence on the formation of such a complex. The experiment was conducted by reacting a solution containing AmpliTaq CS (1 nM) and 5′-(FAM)-labeled 25/36A duplex DNA (1000 nM) with ddTTP alone (400 μM) or in the presence of either dCTP or ddCTP (400 μM each) as the next correct nucleotide. A control experiment consisted of measuring the steady-state rate for ddTTP incorporation (400 μM) in the presence of 400 μM dGTP which represents a "competing" nucleotide that cannot form a correct Watson−Crick base pair in the next template position following ddT polymerization. Figure 5 shows that ddTTP alone turns over with a rate of 0.23 s^{-1} under these reaction conditions and that there was a 50% inhibition in this turnover rate caused by the presence of dCTP (0.12 s^{-1}), which is the next correct deoxynucleotide. The plot also shows that only a correctly base-paired nucleotide causes this inhibition since dGTP showed no effect (0.28 s^{-1}). The simplest interpretation of these observations is that the dissociation rate of the polymerase from the ternary E·DNA_{ddT}·dCTP complex is 50% slower than its dissociation rate from the binary E·DNA_{ddT} complex because the presence of the next correctly base-paired nucleotide causes the polymerase to form a much

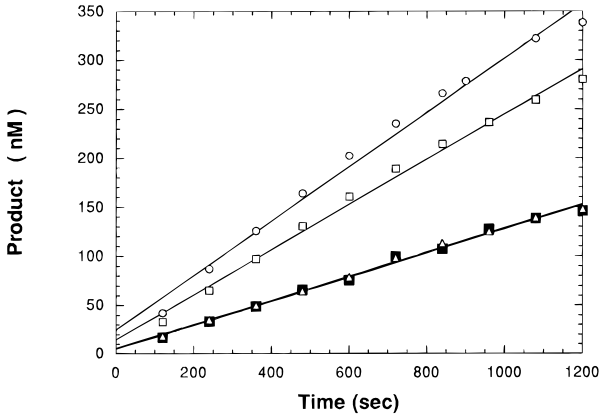

FIGURE 5: Measurement of the dissociation rate for the E·DNA·-Nucleotide complex. A preincubated solution of AmpliTaq CS (1 nM) and 1000 nM 5′-(FAM)-25/36A primer/template was reacted with 2 mM $MgCl_2$ and 400 μM ddTTP alone (□), in the presence of 400 μM dCTP (the next correct deoxynucleotide; ■), in the presence of 400 μM dGTP (incorrect deoxynucleotide; ○), or in the presence of 400 μM ddCTP (the next correct dideoxynucleotide; △). The steady-state rate constants correspond to 0.23 ± 0.007 s^{-1} for dissociation of the binary E·DNA_{dd} complex (ddTTP alone) and to 0.28 ± 0.01 s^{-1} in the presence of another nucleotide that cannot form a correct Watson−Crick base pair in the $n + 1$ position (ddTTP + dGTP). The dissociation rates for the ternary complexes, E·DNA_{dd}·dCTP E·DNA_{dd}·ddCTP were identical, 0.12 ± 0.003 s^{-1} (ddTTP + dCTP) and 0.12 ± 0.004 s^{-1} (ddTTP + ddCTP).

tighter binding complex. This is consistent with a conformational change occurring after nucleotide binding and before the chemistry step. (Taq DNA polymerase contains several tryptophan residues but we have not been able to detect any change in tryptophan fluorescence upon nucleotide binding. Consequently, we do not have any additional evidence to support such a conformational change in Taq DNA polymerase.) Figure 5 also shows that the ddTTP turnover rate in the presence of the next correct dideoxynucleotide, ddCTP, was identical to the ddTTP turnover rate in the presence of dCTP, indicating that a missing 3′-OH group has no effect on the level of inhibition caused by the next correctly base-paired nucleotide. Taken together, these observations showed that a missing 3′-OH group had no affect on ground-state terminator binding nor any affect on the ability of Taq DNA polymerase to form a tighter binding complex, suggesting that a missing 3′-OH group must affect the chemistry step itself.

DISCUSSION

T7 DNA polymerase has been widely used for automated DNA sequencing applications in which the target template DNA is not limited because it yields remarkably uniform peak heights especially in the presence of manganese (Fuller, 1992), but for sequencing applications where the starting template DNA is limited and/or where high throughput is

required, Taq Pol I has become the enzyme of choice. These two DNA polymerases show widely different proficiencies in their abilities to incorporate 2′,3′-ddNTP terminators. Taq Pol I is strongly biased against incorporation of this type of terminator structure, thus necessitating high ddNTP to dNTP molar ratios in typical DNA sequencing reactions (Innis et al., 1988; Tabor & Richardson, 1989). High terminator to substrate ratios and the slow rate of incorporation of terminators could have deleterious effects on DNA sequence traces due, in part for example, to forcing Taq DNA polymerase into a distributive rather than processive mode of synthesis, thus exaggerating any sequence effects on incorporation rates.

In order to better understand how these two polymerases discriminate against 2′,3′-ddNTPs, we chose to measure the basic kinetic constants governing substrate versus terminator incorporation. We also chose to examine a truncated form of Taq Pol I rather than the full-length enzyme because several subtle but practically important advantages have been cited for abbreviated polymerases. For example, truncated forms have been reported to show a 2-fold lower mutation rate in PCR reactions compared to full-length enzymes (Barnes, 1992). Truncated forms have also been shown to be more thermostable but less active than full-length forms above 80 °C (Lawyer et al., 1993). Such paradoxical behaviors might be explained by the inability of truncated forms to polymerize over a mismatch (thereby increasing apparent fidelity) or by the inability to bind DNA at elevated temperatures (thus apparently lowering enzyme activity). Our studies have shown that a truncated form of Taq DNA polymerase, KlenTaq, does indeed bind weakly to synthetic DNA primer/template pairs ($\geq$160 nM) compared to a full-length, exonuclease deficient form, AmpliTaq CS ($\approx$10 nM). Differences in binding and fidelity would be expected to impact sequencing performance, but our goal in this initial study was to measure the basic kinetic parameters that govern dideoxnucleotide terminator incorporation for KlenTaq DNA polymerase during correct nucleotide polymerization under representative DNA sequencing reaction conditions.

Steady-State Kinetics. Our processive steady-state incorporation results showed that KlenTaq strongly preferred dATP over ddATP (about 700 to 1, as shown in Table 2), thus confirming that KlenTaq is just as strongly biased against incorporation of 2′,3′-ddNTP terminators as the full-length, "wild type" enzyme as reported by Tabor and Richardson (1987). We found that the magnitude of this bias could be reduced by using alternative divalent cations. Of the cations tested, however, only manganese showed a differential effect, namely, significantly increased ddAMP incorporation coupled with slightly lower dAMP incorporation. The presence of manganese, however, while increasing the rate of the first incorporation event (as shown by the rate constants in Table 3 which were based upon single-nucleotide incorporation measurements) severely reduced further polymerase activity as shown in Figure 1B, and therefore, the dAMP/ddAMP incorporation ratio could not be reduced below about 17-fold. DNA sequence traces still showed nonuniform peak heights despite this much better incorporation ratio.

Different Rate-Limiting Steps. While processive, steady-state experiments were useful for quickly screening reaction condition effects, they were not sufficient to further dissect the mechanism responsible for this bias. To add further mechanistic details, it was necessary to examine single-nucleotide, turnover kinetics in order to observe individual steps along the forward polymerization pathway. Under conditions where the DNA primer/template was present in 5-fold excess over the enzyme, pre-steady-state patterns showed a burst of Mg^{2+}·dTMP incorporation both for AmpliTaq CS (Figure 2A) and for T7 DNA polymerase (data not shown). It was necessary to use the full-length form of Taq Pol I for these burst experiments because the truncated form, KlenTaq, bound too weakly under these experimental conditions to preform a stable Enz·DNA complex. The fact that there was a burst of Mg^{2+}·dTMP incorporation clearly indicated that the rate-limiting step must occur following the chemistry step in the reaction for naturally occurring substrates for both Taq and T7 DNA polymerases. Additional measurements of the actual enzyme release rate for AmpliTaq CS ($2.4 \pm 0.2\ s^{-1}$) using a DNA "trap" method confirmed that the rate-limiting step for dNTP incorporation was, indeed, due to dissociation of the enzyme from the E·DNA complex. Pre-steady-state experiments for terminator incorporation in the presence of magnesium showed quite dissimilar behavior. Though the bacteriophage enzyme still showed burst kinetics, AmpliTaq CS did not, indicating that for Taq DNA polymerase, the incorporation of terminators was much slower than the enzyme release rate. Therefore, the rate-limiting step for terminator incorporation must occur *after* the chemistry for T7 but *at* or *before* the chemistry for Taq DNA polymerase.

In view of these observations, there are several possible mechanistic explanations for the strong preference that Taq DNA polymerase shows for substrates over terminators. Since the only structural difference between 2′-deoxy and 2′,3′-dideoxynucleotides is a missing 3′-OH group on terminators, this hydroxyl group must be involved in facilitating binding of substrates and/or in aligning reactive groups in the active site for Taq but not for T7 DNA polymerase. Our measurements show that the 3′-OH group is not involved in ground-state substrate binding because the binding constants for dNTPs and 2′,3′-ddNTPs (except for ddATP) were essentially the same ($K_d \approx K_{dd}$). It was the rate of phosphodiester bond formation (k_{pol}) rather than terminator binding that was responsible for much slower terminator incorporation, suggesting that the 3′-OH group on substrates could be involved in stabilizing a transition-state intermediate in the reaction. Stated in terms of transition-state theory, the 3′-OH group could contribute 5.0 kcal/mol to transition-state stabilization, as shown in eq 3, which is large for a single hydrogen bond but not unprecedented (Frick et al., 1989). Such a large apparent transition-

$$\Delta\Delta G^{\ddagger} = -RT \ln\left[\frac{k_{\text{pol-d}}}{k_{\text{pol-dd}}}\right] \qquad (3)$$

state stabilization may be due in part to an effect of the 3′-OH group on the proper alignment of the 5′-phosphate required for catalysis in addition to forming a hydrogen bond. For example, the 3′-OH group may cause the furanose ring to adopt a conformation that favors proper alignment of the reactive groups in the active site. Conversely, the missing 3′-OH group could cause the furanose ring to adopt a conformation that would perturb the alignment of the 5′-phosphate following the formation a tight binding ternary complex with the incoming nucleotide. Thus misalignment

would be expected to retard the rate of phosphodiester bond formation without affecting ground-state binding.

Effect of a Missing 3′-OH Group on the E·DNA·Nucleotide Dissociation Rate. The presence of the next, correct nucleotide has been shown to inhibit the steady-state turnover of HIV RTase under conditions where the chemistry step has been blocked (Kati et al., 1992). Under the same reaction conditions, a nucleotide concentration dependent change in tryptophan fluorescence has also been shown for this polymerase (R. Goody, personal communication). These observations have been interpreted to mean that HIV RTase undergoes a conformational change or isomerization following correct, nucleotide binding and immediately prceeding the chemistry step. A similar conformational change has been shown to be the rate-limiting step in the catalytic pathway for other Pol I type enzymes (Kuchta et al., 1987; Patel et al., 1991) and has been proposed as part of a two-step, substrate binding mechanism that is responsible for the extraordinarily high fidelity of T7 DNA polymerase (Johnson, 1993). According to this model, the first step involves ground-state binding of the substrate through Watson−Crick base pairing with the template and formation of an E·DNA·-dNTP complex. The second step involves an additional check on correctness of base pairing in that only correct pairs can induce a rapid isomerization of the polymerase to form a tighter binding E′·DNA·dNTP complex, which immediately leads to the nucleoside phosphoryl transfer reaction.

The 50% slower dissociation rate shown in Figure 5 was less than the 90% inhibition reported for HIV RTase (Kati et al., 1993); nevertheless, it is consistent with the formation of a tighter binding, ternary complex following correct nucleotide binding. Since both dCTP and ddCTP inhibit to the same extent as shown in Figure 5, Taq DNA polymerase is equally capable of forming a tight-binding complex whether or not the incoming nucleotide has a 3′-OH group. Hence, the missing 3′-OH group on terminators would not be expected to affect any putative conformational change in Taq DNA polymerase, suggesting that the rate-limiting step for terminator incorporation must occur after the formation of a tight-binding, ternary complex. Therefore, the rate-limiting step for terminator polymerization must be the chemical reaction step itself.

Furanose Ring Configuration and Alignment of the Reactive Groups. As mentioned earlier, another plausible mechanism for the strong bias that Taq Pol I shows against 2′,3′-dideoxy terminators is that they may adopt a furanose ring conformation that is unfavorable for rapid phosphodiester bond formation. Sloan et al. (1975) found that when bound to *E. coli* Pol I, Mg^{2+}·dNTPs assumed a 2′-*endo* sugar configuration which is the same form of the furanose ring found in the B-form of DNA. Presumably this would be the preferred ring shape of any transition-state intermediate form in the reaction. NMR studies on 2′-substituted uridines have shown that the electronegativity of the 2′- and 3′-substituents may influence or, perhaps, even determine the preferred conformation of the furanose ring (Guschbauer & Jankowski, 1980). A missing 3′-hydroxyl group on 2′,3′-ddNTPs, therefore, could have a deleterious effect on their incorporation by Taq DNA polymerase through adoption of a 3′-*endo* or less reactive ring configuration.

In this model, the initial collision of substrate results in the formation of a ground-state binding complex which then changes to a tight-binding complex followed by a fast nucleoside phosphoryl transfer reaction. The reaction is fast for dNTPs because the furanose ring is already in a favorable 2′-*endo* configuration. According to this model, 2′,3′-ddNTPs are also capable of forming a tight-binding complex; however, their rate of nucleoside phosphoryl transfer is much slower because their reactive groups are not properly aligned due to an unfavorable configuration of the furanose ring. Replacing the missing 3′-OH group with another electronegative substituent such as fluorine would be expected to restore some of the reactivity of 2′,3′-ddNTPs. Our results showed that 3′-F-dTTP was, indeed, somewhat more reactive ($k_{pol}/K_d \approx 1200\ M^{-1}\ s^{-1}$) than 2′,3′-ddTTP ($k_{pol}/K_d \approx 170\ M^{-1}\ s^{-1}$) but only by ≈7-fold. Although it is supportive, a mere 7-fold improvement for terminator selectivity is still far below the reactivity demonstrated by 2′-dNTPs.

Manganese Effects. Manganese alone or in the presence of magnesium has been shown to significantly affect T7 DNA polymerase performance, yielding remarkably uniform peak heights (Fuller, 1992). A much smaller manganese effect has been shown for Taq DNA polymerase (Tabor & Richardson, 1989). Our observations were consistent with these publications. T7 DNA polymerase showed far less discrimination against 2′,3′-ddNTPs in the presence of magnesium as shown by the apparent second-order selectivity constants listed in Table 4. In the presence of manganese, T7 DNA polymerase was not able to distinguish between substrate and terminator forms, as predicted by a selectivity ratio of one, and its sequence traces showed uniform peak heights. For Taq DNA polymerase, manganese reduced but did not eliminate its ability to distinguish terminators from substrates. Peak height profiles were improved by manganese but were still far from uniform.

In this initial study, we have taken care to minimize any sequence context effects by using a single, consistent primer/template sequence. Our results show that there are differences in the rates of incorporation and binding of the four individual 2′-dNTPs and the four individual 2′,3′-ddNTPs. These differences may contribute to uneven peak heights but are not sufficient, in themselves, to account for the variability observed in DNA traces.

Due to a strong bias against incorporation of 2′,3′-ddNTPs, high terminator to substrate molar ratios are necessary for Taq DNA polymerase in order to drive the incorporation of terminators. In the face of such vast differences in selectivity, even small influences on the rate of terminator incorporation would be expected to have rather dramatic effects on incorporation, resulting in uneven peak height profiles. A major contributing factor must be sequence context as has been shown for the Klenow fragment (Mendelman et al., 1989) for which insertion and DNA dissociation rates have been shown to vary by as much as 7−20-fold depending upon the 5′ nearest neighboring base (Bloom et al., 1993). Nearest neighbor effects have also been proposed to explain uneven peak height patterns generated by Taq DNA polymerase as revealed by heterozygote analysis (Parker et al., 1995). We propose that a strong selectivity bias against terminators is one of the major contributing factors to uneven peak height profiles in DNA sequence traces because it exaggerates sequence dependent differences in incorporation rates especially under conditions where the polymerase would be limited relative to the DNA concentration, such as during the later amplification cycles in cycling−sequencing. The amount of each individual termination fragment or "rung"

on the eventual sequence ladder would be determined by sequence-dependent partitioning among termination, extension, and dissociation events.

Our observations show that Taq, unlike T7 DNA polymerase, requires a 3′-OH group on the incoming nucleotide to support a rapid nucleoside phosphoryl transfer reaction. Taq DNA polymerase is strongly biased against 2′,3′-ddNTP incorporation despite equivalent ground-state binding because this type of terminator reacts slowly relative to 2′-dNTP substrates. Reducing or eliminating a strong bias against terminator insertion would be expected to affect sequencing performance. Two methods can be used to affect selectivity: (1) changes in terminator structure and/or (2) changes in the polymerase. Although they are very preliminary, our initial measurements for alternative terminators such as 3′-F-dTTP suggested that even a modest 7−10-fold reduction in selectivity bias improves peak height profiles. However, since a several thousand-fold reduction in selectivity would be necessary to make Taq DNA polymerase behave more like T7 DNA polymerase, it may be far more rewarding to attempt changing the polymerase through mutagenesis to better incorporate 2′,3′-ddNTPs rather than to attempt improving terminator structure alone.

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NOTE ADDED IN PROOF

During the review of this article, a mutant form of Taq Pol I was described (Tabor & Richardson, 1995) that has a 2′,3′-ddNTP to 2′-dNTP incorporation ratio of unity and also shows remarkably uniform peak height patterns in DNA sequence traces.

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