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Departments of Chemistry and Biochemistry and Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210

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DNA Polymerase β: Pre-Steady-State Kinetic Analysis and Roles of Arginine-283 in Catalysis and Fidelity†

Brian G. Werneburg,‡ Jinwoo Ahn,‡ Xuejun Zhong,‡ Robert J. Hondal,‡ Vadim S. Kraynov,§ and Ming-Daw Tsai*,‡,§,||

Departments of Chemistry and Biochemistry and Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: DNA polymerase β (pol β) is the smallest and least complex DNA polymerase. The structure of the enzyme is well understood, but little is known about its catalytic properties, particularly processivity and fidelity. Pre-steady-state analysis of the incorporation of a single nucleotide into a short 25/45 oligonucleotide primer-template by pol β was used to define the kinetic parameters of the polymerase. In addition, nucleotide analogs and site-specific mutants, along with structural analyses, were used to probe the structure−function relationship of pol β. Several significant findings have been obtained: (i) The catalysis by pol β is processive and displays an initial burst under pre-steady-state conditions, but the processivity is poor compared to other polymerases. (ii) The fidelity of pol β is also low relative to other polymerases. (iii) Under pre-steady-state conditions the chemical step appears to be only partially rate-limiting on the basis of the low thio effect (4.3), defined as $k_{pol(dNTP)}/k_{pol(dNTP\alpha S)}$. The thio effect increases to 9 for incorporation of an incorrect nucleotide. These results are consistent with the existence of a substrate-induced conformational change that is also partially rate-limiting. (iv) A comparison between the two-dimensional NMR spectra of the wild-type and mutant enzymes indicates that the mutations at position 283 did not significantly perturb the structure of the enzyme. The conformational stability of the mutants is also unperturbed. Thus, R283 is not important to the overall structure of the enzyme. (v) The results of kinetic analyses of R283A and R283K mutants indicate that the hydrogen bond between R283 of pol β and the template is important for catalysis. Both R283A and R283K mutants displayed decreases in catalytic efficiency by a factor of *ca.* 200 relative to wild-type pol β. The mutants are also less faithful by a factor of 2−4, in terms of the T-G mispair *vs* the T-A correct pair. The perturbation, however, could occur at both the implied conformational step and the chemical step, since the thio effects of the mutants for both correct and incorrect nucleotides are similar to those of WT pol β.

DNA polymerase β (pol β)[1] performs an essential part of the DNA repair process in filling single-stranded gaps that remain after mutagenic DNA is removed (Weissbach, 1979; Fry, 1983; Singhal & Wilson, 1993). This important DNA repair enzyme was first purified from rats in 1971 (Berger *et al.*, 1971), cloned in 1986 (Zmudzka *et al.*, 1986; Matsukage *et al.*, 1987), and expressed in *Escherichia coli* in 1988 (Abbotts *et al.*, 1988; Date *et al.*, 1988). Studies of template-directed nucleotidyl transfer reactions catalyzed by pol β, the smallest (39 kDa monomer) and the least complex DNA polymerase, are not complicated by auxiliary catalytic functions (*e.g.*, 5′ → 3′ exonuclease, 3′ → 5′ exonuclease, and RNase H) that are often intrinsic to other polymerases. Therefore, pol β could provide a good model for enzyme-catalyzed nucleotidyl transfer reactions in terms of template specificity, metal ion specificity, nucleotide specificity, fidelity, and processivity.

The crystal structure of pol β complexed with DNA, ddCTP, and two divalent cations (Pelletier *et al.*, 1994) suggests that pol β catalyzes DNA synthesis by a divalent cation dependent mechanism, similar to the one proposed for the nucleotidyl transfer reaction (Steitz, 1993). Although the biological relevance of this structure has been brought into question for reasons including the length of the DNA primer and template, the conditions used in crystallization,

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

‡ Department of Chemistry.

§ Department of Biochemistry.

|| Ohio State Biochemistry Program.

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[1] Abbreviations: 25/45mer, 25mer oligonucleotide annealed to a 45mer oligonucleotide as described under Materials and Methods; CD, circular dichroism; 2D, two dimensional; DEAE, diethylaminoethyl; dATP, 2′-deoxyadenosine 5′-triphosphate; dATPαS, 2′-deoxyadenosine 5′-(1-thiotriphosphate); ddCMP, 2′,3′-dideoxycytosine 5′-monophosphate; ddCTP, 2′,3′-dideoxycytosine 5′-triphosphate; dGMP, 2′-deoxyguanosine 5′-monophosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dGTPαS, 2′-deoxyguanosine 5′-(1-thiotriphosphate); dNMP, 2′-deoxynucleoside 5′-monophosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gdn·HCl, guanidine hydrochloride; HIV, human immunodeficiency virus; KF, Klenow fragment of DNA polymerase I; KF(exo⁻), a site-specific mutant of the Klenow fragment with diminished 3′ → 5′ exonuclease activity; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhanced spectroscopy; PCR, polymerase chain reaction; pH*, pH in D_2O without the deuterium isotope effect; pol β, rat DNA polymerase β; RT, reverse transcriptase; S_N2, bimolecular nucleophilic substitution; T4 pol, T4 DNA polymerase; T7 pol, T7 DNA polymerase; T7 pol(exo⁻), a site-specific mutant of T7 DNA polymerase with diminished 3′ → 5′ exonuclease activity; Tris·HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; Tris-d_{11}, perdeuterated 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

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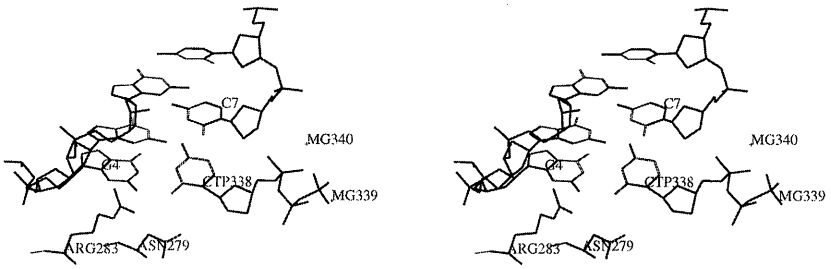


FIGURE 1: Stereoview of the active site of pol β. The stereo diagram was taken from the structure solved by Pelletier *et al.* (1994). Only two of the base pairs between the primer and template DNA are shown. The primer is terminated at the 3′-end by ddCMP (C7). R283 hydrogen bonds with dGMP (G4) of the template, which forms three hydrogen bonds with the incoming nucleotide, ddCTP (CTP338). N279 hydrogen bonds with ddCTP. The two catalytic metal ions designated MG339 and MG340 are coordinated by ddCTP.

and potential distortion due to crystal packing forces (Arnold *et al.*, 1995), this pseudoternary complex crystal structure is the only structure of a ternary complex of a nucleotide polymerase available to date. The crystal structures of other DNA and RNA polymerases including Klenow fragment (Ollis *et al.*, 1985; Beese *et al.*, 1993a,b), HIV RT (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993), T7 RNA polymerase (Sousa *et al.*, 1993), and *Thermus aquaticus* DNA polymerase (Kim *et al.*, 1995) have been solved, but none of them contain analogs of both the DNA and dNTP substrates or a transition-state analog.

While pol β is the best understood polymerase in terms of structure, it is one of the least understood in terms of kinetics. Pre-steady-state kinetic analysis, which has been used to study several polymerases, has not been performed on pol β. Steady-state kinetic studies have provided preliminary information pertaining to the kinetic scheme. Steady-state analyses demonstrate that the catalytic rates of pol β on poly(rA) and poly(dA) homopolymer substrates are similar to those of KF (Abbots *et al.*, 1988). However, in contrast to KF, which catalyzes processive DNA polymerization on synthetic oligonucleotide template-primer substrates (Kuchta *et al.*, 1987), distributive catalysis by pol β was observed with synthetic oligonucleotide DNA substrates; after each incorporation of dNMP, the enzyme·DNA complex dissociated (Singhal & Wilson, 1993; Prasad *et al.*, 1994). Pol β does have greater affinity for DNA templates with short single-stranded gaps (Prasad *et al.*, 1994), suggesting that it can synthesize DNA processively under certain conditions.

The frequencies of misincorporation by pol β have been estimated by forward mutation assay to be the highest of all eukaryotic DNA polymerases (Kunkel & Alexander, 1986). Misincorporation frequencies of HIV RT (Kati *et al.*, 1992), known for its infidelity, are comparable to the error frequencies of pol β; the most commonly made mistake by both enzymes is the G-T mispair which is made once for every 2000 correct A-T base pairs.

Steady-state analyses are not sufficient for understanding the detailed mechanism of a polymerase, since steady-state rates are averages of the rates for polymerization and the dissociation of enzyme·DNA complex. Pre-steady-state analyses were essential in defining the kinetic schemes of the reactions catalyzed by KF (Kuchta *et al.*, 1987), T7 pol (Patel *et al.*, 1991), HIV RT (Kati *et al.*, 1992; Reardon, 1992), and T4 pol (Capson *et al.*, 1992). In addition, while the forward mutation assay for misincorporation provides a good estimate of the error frequency, a comparison of the pseudo-first-order rate constants for incorporation of correct and incorrect nucleotides would provide a direct measurement of the error frequency (Wong *et al.*, 1991).

The first pre-steady-state analysis of single turnovers catalyzed by pol β is described in this report. The results indicate that the reaction catalyzed by pol β is processive. The rate of catalysis and processivity achieved by pol β are comparable to those exhibited by KF (Kuchta *et al.*, 1987) and HIV RT (Kati *et al.*, 1992; Reardon, 1992). The fidelity of DNA replication determined kinetically, in terms of the T-G mispair *vs* the T-A correct pair, is similar to that previously measured by forward mutation assay (Kunkel & Alexander, 1986).

Furthermore, the plausible substrate-induced conformational change step, which has been shown to be slower than the chemical step in other polymerases (Johnson, 1993; Kati et al., 1992; Frey et al., 1995), was probed by using phosphorothioate analogs of dNTP. The results suggest that catalysis by pol β may also involve a conformational step, the rate of which is comparable to the rate of the chemical step.

In addition to the analyses of wild-type pol β, site-directed mutagenesis, pre-steady-state kinetics, NMR, and CD were used to study the roles of Arg-283 in fidelity, catalysis, global conformation, and conformational stability. Arg-283 forms a hydrogen bond with the base of the template that hydrogen bonds with the incoming dNTP substrate (Pelletier *et al.*, 1994) (Figure 1). The results suggest that Arg-283 is important for catalysis but not for the structure of the enzyme.

MATERIALS AND METHODS

Materials. All oligonucleotides were purchased from Bio-Synthesis Inc. All dNTPs were purchased from Pharmacia-LKB except for S_p-dATPαS and S_p-dGTPαS, which were purchased from United States Biochemicals. Sprague-Dawley rat brain (*ca.* 1 week old) was purchased from Harlan Bioscience Products. An mRNA isolation kit was purchased from Stratagene, and an RT PCR kit was purchased from Perkin-Elmer. The pCRII vector from Invitrogen was used for cloning the RT PCR product, M13mp19 phage DNA was used for mutagenesis, and pET-17b from Novagen was used for expression of pol β. HB101 and BL21(DE3)pLysS strains were used as hosts for cloning and expression. *E. coli* strains CJ236 and JM101 were used for mutagenesis. All *E. coli* strains were purchased from Stratagene except BL21(DE3)pLysS (Novagen). All DNA-modifying enzymes were purchased from New England Biolabs, and all other reagents were of the highest purity available commercially.

Synthesis of the Pol β cDNA. The cDNA of pol β was synthesized and amplified by RT PCR of mRNA isolated from 1-week-old Sprague-Dawley rat brain. The sequences of the upstream and downstream primers used for RT PCR

were 5′-TACGCGCATATGAGCAAACGCAAGGCGCC-3′ and 5′-GCGCTAGGTACCTCATTCACTCCTGTCCTTGG-3′, respectively; they were designed using the sequence of the gene encoding pol β (Matsukage *et al.*, 1987). The RT PCR product was ligated directly into pCRII, which had 5′-TT overhangs, and subsequently subcloned into M13mp19 at the *Kpn*I and *Xba*I sites of the phage DNA for mutagenesis. The wild-type cDNA was subcloned into pET-17b at the *Kpn*I and *Nde*I sites of the expression vector.

Expression and Purification of Pol β. The cDNA for pol β was cloned into pET-17b downstream from the T7 promoter as described above. Expression of recombinant pol β in BL21(DE3)pLysS was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (A_{600} nm = 0.5) in Rich media (19 g of yeast extract, 12 g of tryptone, and 10 mM $MgSO_4$) containing ampicillin, 100 μg/mL, and chloramphenicol, 34 μg/mL, at 37 °C. After sonication of the BL21-(DE3)pLysS cells containing pol β, the protein extract was passed through DEAE-cellulose to remove nucleic acids before purification. The WT and mutant enzymes were then purified by ion-exchange chromatography on phosphocellulose, affinity chromatography on DNA−cellulose, and gel filtration on either Sephacryl-S100 or Sephacryl-S200, as described by Date *et al.* (1988). The resulting protein was >95% pure as determined by polyacrylamide gel electrophoresis. No exonuclease activity was detected upon incubation of the enzyme with ^{32}P duplex DNA substrate and Mg^{2+} for 10 h (under the conditions described in the next section), indicating that pol β had been successfully separated from *E. coli* DNA polymerase I.

The protein concentration was determined at A_{280nm} with an extinction coefficient of 21 200 M^{-1} cm^{-1} (Casas-Finet *et al.*, 1991), while the enzyme activity was measured in a crude assay at 25 °C with 50 mM Tris·HCl at pH 7.7 containing 120 mM KCl, 0.1 mM DTT, 2.5 mM $MgCl_2$, 40 μg/mL poly(A)−p$(dT)_{12-18}$, 0.1 mM [^{3}H]dTTP (200 dpm/pmol), and 15% glycerol (Date *et al.*, 1988). After the addition of 1 μL of enzyme solution to 25 μL of assay mixture, aliquots were removed as a function of time, quenched with 0.3 M EDTA, and added to DEAE filter paper discs (DE-81). The filter paper was washed for 5 min with 0.3 M sodium formate and 5 min with EtOH before being dried and counted in scintillation cocktail to determine the amount of [^{3}H]dTMP incorporated as a function of time. The specific activity of the purified enzyme as measured under these conditions was 1.0 × 10^5 units/mg. One unit of enzyme incorporated 1 nmol of dTMP/h.

Rapid-Chemical-Quench Experiments. These experiments were performed with an apparatus designed by K. A. Johnson (1986) and built by KinTek Instruments Corp. The duplex DNA substrate (25/45mer) used in this study, an oligonucleotide primer (5′-GCCTCGCAGCCGTCCAACCAACTCA, 25mer) annealed to an oligonucleotide template (3′-CG-GAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTT-ACGGCAGG, 45mer), was identical to the substrate used for rapid-quench studies of HIV RT (Kati *et al.*, 1992) and similar to that used in a similar study of T7 pol (Patel *et al.*, 1991).

Both oligonucleotides were purified by electrophoresis through denaturing (7 M urea) polyacrylamide gels; a 16% gel was used to purify the 25mer and a 10% gel for the 45mer. The 25mer was 5′-radiolabeled (1000−5000 dpm/pmol) with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maniatis *et al.* (1982) and annealed to the 45mer as described by Patel *et al.* (1991). Pol β was preincubated with the DNA substrate prior to rapid mixing with dNTP and divalent cation to begin the reaction that was quenched with 0.3 M EDTA. The typical experiment was performed at 37 °C in 50 mM Tris·HCl, pH 7.7, containing 50 mM KCl, 10% glycerol, 0.1 mg/mL bovine serum albumin, 1 mM DTT, 100 nM pol β, 200 nM 25/45mer DNA duplex, 200 μM dNTP, and 500 μM $MnCl_2$ or 2.5 mM $MgCl_2$. Product formation was monitored as a function of time with a Beta Scope from Betagen after denaturing (7 M urea) polyacrylamide gel electrophoresis was used to separate 26mer product from 25mer substrate.

On the basis of the protein concentrations determined by absorbance at A_{280nm} with an extinction coefficient of 21 200 M^{-1} cm^{-1} (Casas-Finet *et al.*, 1991), and the active site concentrations determined by burst height in the pre-steady-state kinetic studies, 60−90% of the wild-type and mutant pol β protein converted substrate into product. This phenomenon has also been observed with HIV RT where 50% of the active sites were active (Kati *et al.*, 1992). When bovine serum albumin was not included in the reaction mixture, only 30% of the protein catalyzed dNTP incorporation. The concentrations of the stabilizing agents, bovine serum albumin and glycerol, in the reaction mixture were not increased beyond those described above, because higher concentrations could affect the viscosity of the mixture causing an increase in the mixing times during fast quench. The concentrations of pol β reported under Results were determined by absorbance at 280 nm.

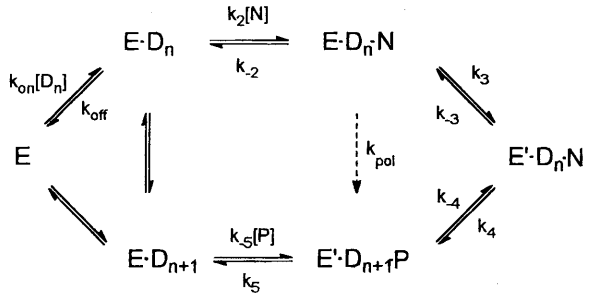
DNA Trapping Experiment. A direct measurement of k_{off} was achieved with a trapping experiment similar to one described by Patel *et al.* (1991). Pol β·25/45mer complex was incubated with 0.1 mg/mL salmon sperm DNA (0−60 s) before the addition of MgdATP to begin the reaction. Each reaction was quenched after 10 s with 0.3 M EDTA. Product formation was plotted as a function of the incubation time with the DNA trap. The decrease in product formed *vs* time was fit with an exponential equation to determine k_{off}.

Site-Directed Mutagenesis. R283A and R283K were created by the method of Kunkel (1985). R283K was constructed from the oligonucleotide 5′-GGGCATGCGC-TTTCATATTCTTATTAAA, where the underlined base represents the mutated site. The oligonucleotide used for the construction of R283A was 5′-GGGCATGCGCTGC-CATATTCTTATTAAA. The resulting mutants were verified by sequencing the entire pol β gene (Sanger *et al.*, 1985). The mutants were subcloned into pET-17b as described above.

Gdn·HCl-Induced Denaturation and Conformational Stability. CD spectra were recorded on a JASCO J-500C spectropolarimeter using a thermostated quartz microcell and processed using DP-500/AT system (version 1.29) software. A stock solution of 7.4 M Gdn·HCl was prepared with double distilled water, and the exact concentration was determined by refractive index (Nozaki, 1972). Samples, which contained 0.2 mg/mL enzyme, were prepared in 5 mM borate at pH 8.0 containing 50 mM KCl and various concentrations of Gdn·HCl. The samples were incubated at 22 °C for 10 min and then scanned five times from 250 to 200 nm. The ellipticity at 222 nm was recorded and used to determine the free energy of unfolding, $\Delta G_d^{H_2O}$.

2D NMR of Pol β and Mutants. All NMR experiments were performed on a Bruker DMX-600 spectrometer.

Scheme 1: Postulated Kinetic Scheme for Pol β[a]



[a] D_n, N, and P represent DNA, dNTP, and inorganic pyrophosphate, respectively.

Chemical shifts were referenced to sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$.

Pol β samples were purified as described above except for the following differences. Chromatography on the phosphocellulose column was performed in the absence of glycerol. The fractions containing pol β were pooled and precipitated with ammonium sulfate (52 g/100 mL). After centrifugation for 2 h at 14 000*g*, the pellet was dissolved in 10 mL of dialysis buffer (50 mM ammonium bicarbonate at pH 7.6 containing 0.04 mM EDTA and 0.1 mM DTT) and dialyzed for 8 h. The samples were then lyophilized and stored at −70 °C. The samples were exchanged with D_2O for 10 min and lyophilized again. The final concentration of enzyme in the NMR sample was 0.3 mM in D_2O, containing 40 mM Tris-d_{11}, 130 mM KCl, 0.04% NaN_3, and 1 mM DTT. The sample also contained residual ammonium bicarbonate from the last purification step. The pH was adjusted to 7.5 without isotope correction. The NMR spectra were recorded at 37 °C.

Standard pulse sequence and phase cycling were used for the NOESY experiments (Bodenhausen *et al.*, 1984), with a mixing time of 100 ms. All spectra were obtained in the phase-sensitive mode using TPPI and with water suppression using a 3−9−19 pulse sequence with gradients (Piotto *et al.*, 1992; Sklenar *et al.*, 1993). The sweep widths were 13 ppm. A total of 2048 × 416 matrices in the time domain were recorded and zero-filled to 2048 × 1024 matrices prior to multiplication by a Gaussian function (LB2 = −3, GB2 = 0.05) in the f_2 dimension and shifted sine bell (SSB1 = 8) in the f_1 dimension and Fourier transformation.

RESULTS

Cloning, Expression, and Purification. Pol β cDNA, which has been cloned previously by immunological screening of a λgt11 cDNA library (Zmudzka *et al.*, 1986), was cloned directly here from mRNA by RT PCR. Rat brain mRNA was reverse transcribed and amplified by RT PCR to generate a sufficient quantity of pol β cDNA, which was then cloned into the pET-17b expression vector. Pol β was overexpressed in BL21(DE3)pLysS cells; approximately 20% of the total protein in the cells after induction was pol β. Pol β was purified according to the procedure of Date *et al.* (1988).

Processive DNA Synthesis. Although most DNA polymerases catalyze processive synthesis as shown in Scheme 1, the mechanism of pol β appeared to be distributive on the basis of steady-state kinetic analysis (Tanabe *et al.*, 1979). The DNA template binds before dNTP in an ordered fashion and dissociates from pol β after pyrophosphate is released. While structural studies indicate that dNTP may bind to free pol β (Davies *et al.*, 1994; Sawaya *et al.*, 1994), the steady-state kinetic data indicate that the pol β·dNTP complex is not catalytically competent (Tanabe *et al.*, 1979).

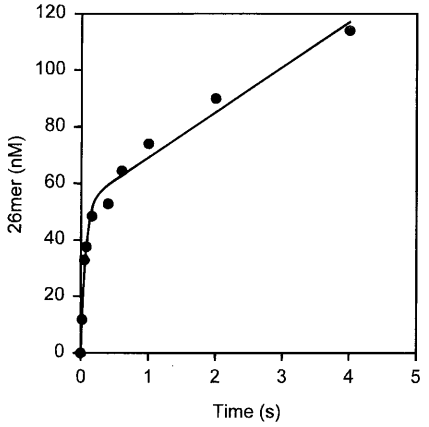


FIGURE 2: Product *vs* time plot of incorporation of a single dAMP nucleotide. The reaction conditions and methods of product analysis were as described under Materials and Methods. Pol β, 90 nM, was incubated with 200 nM 25/45mer and rapidly mixed at 37 °C with 100 μM MndATP to begin the reaction. The data were fit to a burst equation (1) as described in the text.

Table 1: Comparison of the Processivity of Pol β with That of Other Polymerases

enzyme	k_{pol} (s^{-1})	k_{off} (s^{-1})	k_{pol}/k_{off}
pol β (Mg^{2+})	10	0.3	30
pol β (Mn^{2+})	23	0.3	80
T7 pol[a]	300	0.2	1500
T4 pol[b] (*in vivo*)	250	0.3	830
T4 pol[c] (*in vitro*)	400	6	70
KF[d]	50	0.2	250
HIV RT (DNA substrate)[e]	30	0.16	190

[a] Patel *et al.* (1991). [b] Mace and Alberts (1984). [c] Capson *et al.* (1992). [d] Kuchta *et al.* (1987). [e] Kati *et al.* (1992).

We found that pol β catalyzes processive DNA synthesis. Under pre-steady-state conditions the product *vs* time plot (Figure 2) for the incorporation of dAMP into the 25/45mer by pol β was characteristic of a DNA polymerase that is processive on single-stranded DNA templates (Kutchta *et al.*, 1987; Patel *et al.*, 1991; Kati *et al.*, 1992). The height of the burst was equivalent to the concentration of enzyme active sites in the reaction mixture, because the rate of the second turnover was much slower than that of the first. The data were fit to a burst equation:

$$26\text{mer} = A(1 - e^{-k_{obs}t}) + mt \qquad (1)$$

where A, k_{obs}, and m represent burst amplitude, the rate constant for the burst, and the rate of steady-state incorporation, respectively. The steady-state rate constant, defined as k_{ss}, can be obtained by dividing the slope of the slow linear phase of the plot by the enzyme concentration. Fitting of the data in Figure 2 gave $k_{obs} = 15 \pm 3$ s^{-1} and $k_{ss} = 0.3$ s^{-1}.

The pre-steady-state turnover number of pol β, k_{pol} (see Scheme 1), can be obtained by determining k_{obs} at saturating dATP. In actuality, it was determined under a condition with 25/45mer as the limiting reagent, so that the slow phase in Figure 2 is eliminated, as described in a later section on fidelity. The k_{pol} values of pol β and other polymerases are listed in Table 1.

The rate constant for DNA dissociation, k_{off} (see Scheme 1), should be the same as the rate constant for the steady-

state incorporation, k_{ss}, since the steady-state rate is limited by DNA dissociation after the burst. This has been verified by an independent determination of k_{off} by a trapping experiment where the pol β·25/45mer complex was incubated with salmon sperm DNA for 0−60 s before the addition of MgdATP to begin the reaction (data not shown). The decrease in product formed *vs* time of incubation with the trap was exponential. The data were fit to a single exponential equation to determine the off-rate constant for the 25/45mer substrate. The k_{off} thus obtained (0.3 s^{-1}) was the same as k_{ss}.

The ratio between k_{pol} and the off-rate constant, k_{pol}/k_{off}, defines processivity, which represents the average number of nucleotides that pol β would incorporate before dissociating from the DNA template if the template were long enough and all four dNTP substrates were present. Table 1 lists the k_{pol}, k_{off}, and processivity for pol β and several other polymerases. As shown in Table 1, the processivity of pol β on single-stranded DNA template is less than the processivities of T7 pol and T4 pol but comparable to that of KF and HIV RT.

A direct relationship between the dissociation rate constant for the pol β·DNA complex and the monovalent cation concentration was observed. As the K^+ concentration was raised from 50 to 120 mM, k_{off} increased from 0.3 to 0.8 s^{-1}. Thus, the processivity of pol β is related to the monovalent cation concentration. This is logical because the formation of ionic bonds between K^+ ions and the phosphoryl groups of the DNA template could attenuate interactions between the phosphoryl groups and protein residues necessary for tighter binding.

Dissociation Constant of the Pol β·DNA Complex. As described above, the slow dissociation rate of the pol β·26/45mer complex resulted in the accumulation of this complex under conditions where only one correct nucleotide, dATP, could be incorporated into each 25/45mer substrate (Figure 2). The amplitude of the burst in the product *vs* time plot was equivalent to the concentration of the pol β·DNA complex. Therefore, it was possible to titrate the enzyme active sites with 25/45mer by keeping the concentrations of enzyme and MgdATP constant and varying the DNA substrate concentration. Product *vs* time curves were generated at various DNA concentrations, and the concentrations of the pol β·DNA complexes formed were determined by extrapolation from the slow linear phases of the product *vs* time curves to the product ordinate. The dissociation constant of the pol β·25/45mer complex was determined from a plot of the concentration of the pol β·DNA complex *vs* initial DNA concentration as shown in Figure 3. The data were fit to a quadratic equation:

$$[E\cdot D] = 0.5([E]_0 + [D]_0 + K_d) - [0.25([E]_0 + [D]_0 + K_d)^2 - [E]_0[D]_0]^{0.5} \quad (2)$$

where [E·D] is the concentration of the pol β·DNA complex and $[E]_0$ and $[D]_0$ are the initial concentrations of pol β and 25/45mer, respectively. The K_d determined from such analysis was 49 ± 12 nM.

Fidelity and Divalent Cation Specificity. At fixed concentrations of pol β and 25/45mer, the concentration of dATP was varied to generate a series of single exponential product *vs* time plots; the second linear phase of product formation observed in Figure 1 was eliminated by making 25/45mer the limiting reagent ([DNA] < [enzyme]). The observed

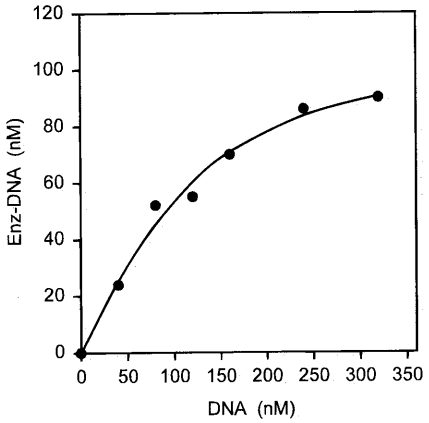


FIGURE 3: Determination of K_d by titration of the enzyme active sites with 25/45mer. The reaction conditions and methods of product analysis were as described under Materials and Methods. Pol β, 150 nM, was incubated with 40, 80, 125, 160, 240, and 320 nM 25/45mer prior to rapid mixing with 100 μM MgdATP at 25 °C in the absence of bovine serum albumin. The product *vs* time plots at each concentration of 25/45mer were plotted as described in Figure 2. The pol β·DNA concentrations that were equal to the burst heights were determined by extrapolation to the *y*-axis. The dissociation constant of the pol β·DNA complex was determined by plotting pol β·DNA ([E·D]) *vs* the initial concentration of 25/45mer ($[D]_0$) and fitting the data to eq 2.

rate constants (k_{obs}) were determined at each concentration of dATP by fitting the data to a single exponential rate equation. The observed rate constants were then plotted against [dATP], and the data were fit to the Michaelis−Menten equation to determine k_{pol} and K_d^{app} for dATP (Figure 4):

$$k_{obs} = k_{pol}[dNTP]/([dNTP] + K_d^{app}) \quad (3)$$

The K_d^{app} is the concentration of dNTP that gives the half-maximal rate of catalysis. The equilibrium dissociation constant, K_d, for the pol β·dNTP complex has not yet been determined.

Since it is known that Mn^{2+} affects the activity and fidelity of certain polymerases including Klenow fragment (Eger *et al.*, 1991), we measured the activity and fidelity of pol β in the presence of manganese. The concentrations of Mn^{2+} and Mg^{2+} required for maximal activity, 0.5 and 2.5 mM, respectively, were determined under pre-steady-state conditions where the enzyme concentration was in 3-fold excess over the DNA concentration. These results agreed with those of Tanabe *et al.* (1979) under steady-state conditions (0.5 and 2.0 mM for Mn^{2+} and Mg^{2+}, respectively). The k_{pol} for the incorporation of dATP was 2-fold greater in the presence of Mn^{2+} than in the presence of Mg^{2+} (Table 2).

An incorrect nucleotide dGTP, which forms a non-Watson−Crick-type base pair with dTMP of the template, was incorporated much more slowly than the correct nucleotide with either Mg^{2+} or Mn^{2+} cofactors. No burst of dGMP incorporation was observed when 25/45mer was in excess over the enzyme, suggesting that the observed rate of incorporation of the incorrect nucleotide was slower than the dissociation rate constant of the pol β·DNA complex. The observed rate constant, k_{obs}, for incorporation of one incorrect nucleotide, dGTP, could be determined as described for the experiments with dATP under the condition where the enzyme was in 3-fold excess over DNA. The Michaelis−Menten equation was used to determine k_{pol} and K_d^{app} for dGTP (Table 2).

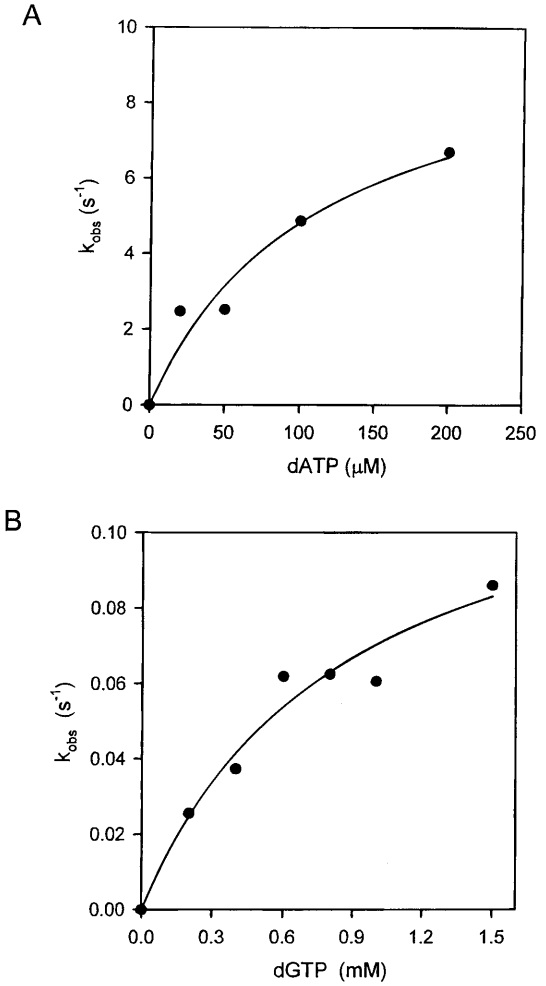


FIGURE 4: Michaelis–Menten plots with the correct and incorrect dNTP. The reaction conditions and methods of product analysis as were described under Materials and Methods. (A) Product *vs* time plots were generated with 20, 50, 100, and 200 μM MgdATP in the presence of 200 nM pol β and 200 nM 25/45mer. The data were fit to the single exponential equation 26mer = $A(1 - e^{-k_{obs}t})$ to determine k_{obs} at each concentration of dNTP. The observed rate constants were plotted *vs* [dATP] to determine k_{pol} (10 ± 3 s^{-1}) and K_d^{app} (110 ± 60 μM). (B) Product *vs* time plots were generated with 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 mM MgdGTP in the presence of 180 nM pol β and 60 nM 25/45mer. The observed rate constants were plotted *vs* [dGTP] to determine k_{pol} (0.13 ± 0.02 s^{-1}) and K_d^{app} (0.85 ± 0.32 mM).

The specificity constants, or apparent second-order rate constants, for the correct $[(k_{pol}/K_d^{app})_c]$ and incorrect $[(k_{pol}/K_d^{app})_i]$ nucleotides were used to determine the fidelity of DNA replication, in terms of the T-G mispair *vs* the T-A correct pair, using eq 4 (Wong *et al.*, 1991):

$$\text{fidelity} = \text{error frequency}^{-1} = [(k_{pol}/K_d^{app})_c + (k_{pol}/K_d^{app})_i]/(k_{pol}/K_d^{app})_i \quad (4)$$

The fidelity of pol β under various conditions is also included in Table 2. The data in Table 2 suggest that pol β can select for the correct T-A base pair over the incorrect T-G base pair at the dNTP binding step; MgdATP bound eight times more tightly to the pol β·25/45mer complex than MgdGTP as determined from the K_d^{app}'s for the two nucleotides. Upon replacement with manganese, fidelity decreased by a factor of 4. A sharp decrease in the K_d^{app} for the incorrect nucleotide, from 850 to 46 μM dGTP, coincides with the decrease in fidelity of DNA synthesis when Mn^{2+} is substituted for Mg^{2+}. This suggests that, in the presence of Mn^{2+}, pol β loses the ability to discriminate between correct, dATP, and incorrect, dGTP, nucleotide substrates at the nucleotide binding step.

Table 2: Fidelity of Wild-Type Pol β and Mutants[a]

enzyme	metal ion	dNMP:dNTP	k_{pol} (s^{-1})	K_d^{app} (μM)	fidelity[b]
WT	Mg^{2+}	T:A	10 ± 3	110 ± 60	
WT	Mg^{2+}	T:G	0.13 ± 0.02	850 ± 320	610
R283K	Mg^{2+}	T:A	0.05 ± 0.01	170 ± 60	
R283K	Mg^{2+}	T:G	0.0013 ± 0.0002	1300 ± 270	290
R283A	Mg^{2+}	T:A	0.048 ± 0.004	64 ± 13	
R283A	Mg^{2+}	T:G	0.0014 ± 0.0001	580 ± 11	280
WT	Mn^{2+}	T:A	23 ± 3	58 ± 12	
WT	Mn^{2+}	T:G	0.13 ± 0.01	46 ± 5	140
R283K	Mn^{2+}	T:A	1.4 ± 0.3	120 ± 50	
R283K	Mn^{2+}	T:G	0.011 ± 0.001	180 ± 40	200
R283A	Mn^{2+}	T:A	0.025 ± 0.003	7.4 ± 5	
R283A	Mn^{2+}	T:G	0.018 ± 0.001	180 ± 30	34

[a] The methods described under Figure 4 were used to determine k_{pol} and K_d^{app} in the presence of a 3–5-fold excess of enzyme over 25/45 mer for the incorporation of the incorrect nucleotide and all of the mutants. The ratio of enzyme to DNA was 1:1 in the reaction mixture for assay of correct nucleotide incorporation by wild-type enzyme. [b] The fidelity of DNA replication (error frequency^{-1}) is equal to $[(k_{pol}/K_d^{app})_c + (k_{pol}/K_d^{app})_i]/(k_{pol}/K_d^{app})_i$.

Structural Analysis of R283A,K Mutants. Arg-283 has been observed to hydrogen bond directly with the template base that hydrogen bonds with the nitrogenous base of the incoming nucleotide (Pelletier *et al.*, 1994), as shown in Figure 1. The residue was changed to alanine and lysine to test the importance of this residue in the structure and catalysis of pol β. Proton NMR and Gdn·HCl-induced denaturation were then used to examine the changes in conformation and conformational stability, respectively.

Although the molecular weight of pol β (39 000) is large for detailed NMR analysis, we have been able to obtain 1D and 2D NOESY spectra that are good for qualitative comparison between WT and mutants. As shown in Figure 5, the NOESY spectra of wild-type pol β, R283A, and R283K are similar except for a few minor chemical shift differences. The results indicate that the conformations of R283A and R283K are not globally perturbed.

The conformational stability of WT and the mutants was measured by Gdn·HCl-induced denaturation, monitored by CD spectroscopy. The denaturation curves, shown in Figure 6, display a behavior consistent with a two-state folding mechanism. The denaturation data were fit with the following equation (Pace, 1986):

$$\Delta G_d = \Delta G_d^{H_2O} - m[\text{Gdn·HCl}] \quad (5)$$

where ΔG_d is the Gibbs free energy change of denaturation at various concentrations of guanadine·HCl, $\Delta G_d^{H_2O}$ is that at zero concentration of Gdn·HCl, and m is a constant related to the susceptibility of the enzyme toward denaturation by the denaturant. The $\Delta G_d^{H_2O}$ values, the midpoint of the denaturation curve ($D_{1/2}$), and the slope (m) are listed in Table 3. The data suggest that the mutations at position 283 did not perturb the conformational stability.

Functional Analysis of R283A,K Mutants. No burst of activity was observed in the reactions catalyzed by either mutant (except for R283K in the presence of Mn^{2+}), because the rate of incorporation of the first nucleotide was slower than the off-rate for the DNA template. The observed rate

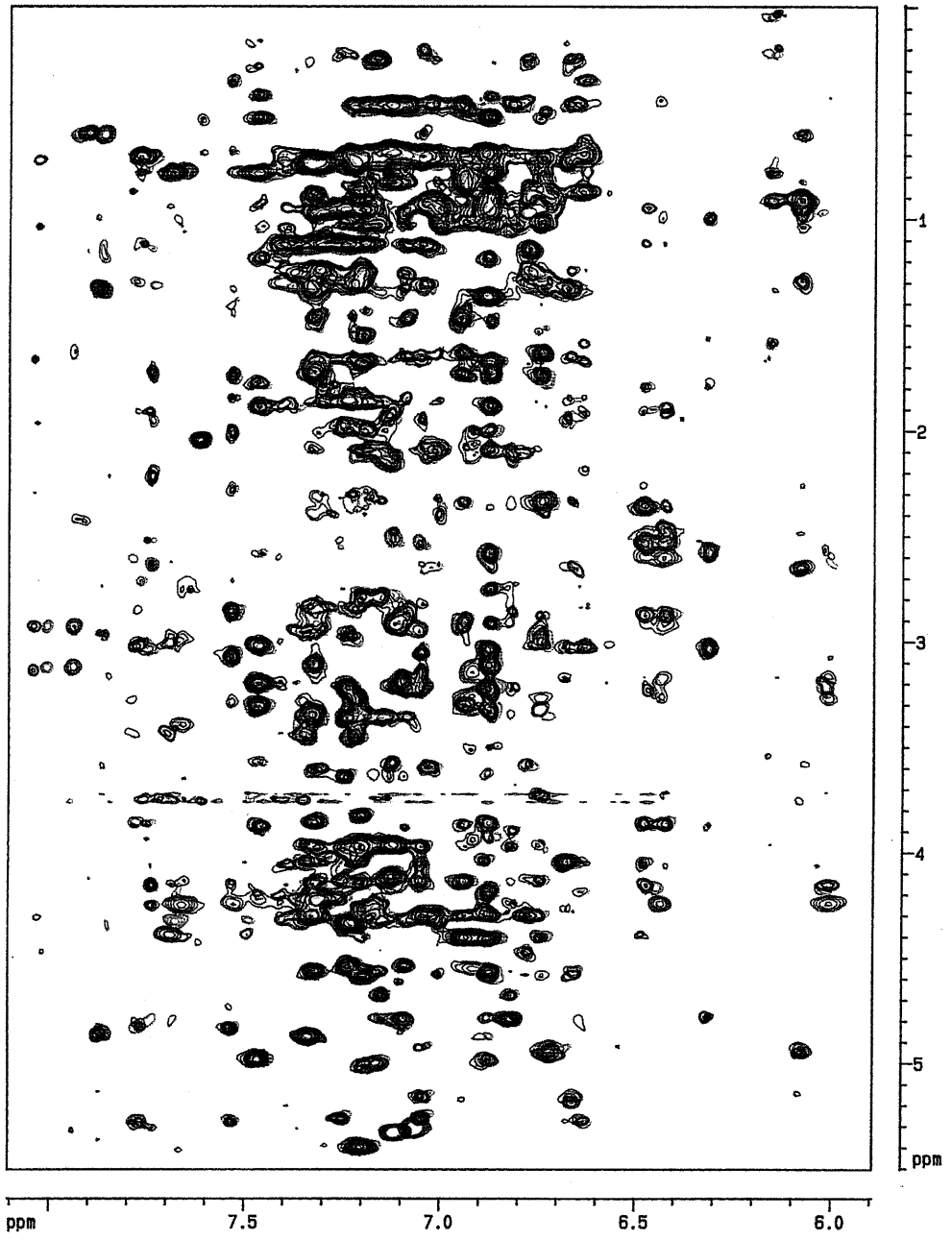


FIGURE 5: Aromatic−aliphatic interresidue NOEs in phase-sensitive NOESY spectra of pol β (green), R283K (yellow), and R283A (red). The NMR samples used to generate the spectra contained 0.3 mM enzyme in D_2O at 37 °C, pH 7.5. Mixing times were 100 ms.

Table 3: Free Energy of Gdn·HCl-Induced Denaturation for Wild-Type Pol β and Mutants[a]

enzyme	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m [kcal/(mol·M)]
WT	2.3	1.2	2.0
R283K	2.6	1.2	2.4
R283A	2.7	1.2	2.3

[a] The error limit in $\Delta G_d^{H_2O}$ is estimated to be ±0.5 kcal/mol.

constants for the first turnovers of the mutants were determined with the concentrations of the mutant enzymes in 3−5-fold excess over the concentration of the DNA template, and the results are shown in Table 2. The rate of catalysis was significantly reduced upon mutation to either alanine or lysine, suggesting that the hydrogen bond between R283 and the template base is important for catalysis. Thus, while R283 is not important to the overall structure of the enzyme, it is important for its catalytic function.

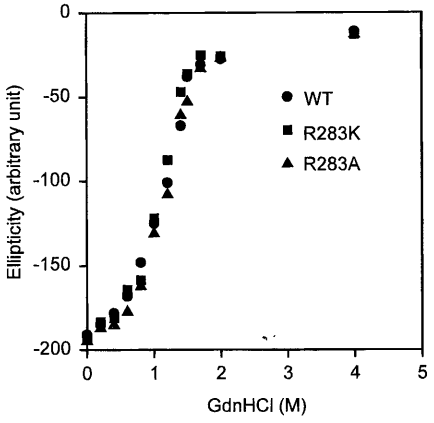


FIGURE 6: Denaturation curves for WT, R283K, and R283A at various Gdn·HCl concentrations. The solutions containing 5 mM borate, 50 mM KCl, pH 8.0, and enzymes were incubated at 22 °C for 10 min and then scanned five times from 250 to 200 nm. The ellipticity at 222 nm was recorded.

Table 4: Summary of the Thio Effect for WT and Mutants[a]

enzyme	dNMP:dNTP	$k_{pol(dNTP)}$	$k_{pol(dNTP\alpha S)}$	thio effect
WT	T:A	10	2.3	4.3
WT	T:G	0.45	0.05	9
R283K	T:A	0.05	0.016	3.1
R283K	T:G	0.00085	7.6×10^{-5}	11
R283A	T:A	0.048	0.017	2.8
R283A	T:G	0.00084	9.9×10^{-5}	9

[a] The k_{pol} values for dNTP and dNTPαS were obtained at saturating nucleotide concentrations ($5 \times K_d^{app}$ of dATP). The k_{pol} values for incorrect nucleotides (which have higher K_d^{app}) were obtained at 1 mM for both dGTP and dGTPαS, since use of higher concentrations could lead to substrate inhibition. Thus for the incorrect nucleotides, k_{pol} is actually k_{obs} at a fixed concentration.

Multiple bonds between pol β and the DNA substrate, including the hydrogen bond between R283 and the template, contribute to the enzyme's affinity for the DNA substrate (Pelletier *et al.*, 1994). It is therefore important to determine if the loss in catalytic efficiency of the R283A and R283K mutants could be attributed in part to decreased affinity for the DNA substrate. The dissociation constants for the mutant pol β·DNA complexes could not be determined in the presence of Mg^{2+}, because $k_{pol} < k_{off}$ and no pre-steady-state burst in activity was observed. However, when the DNA substrate was in excess over the R283K mutant, a burst was observed in the presence of Mn^{2+}. The steady-state rate constant, k_{ss}, which was demonstrated to be equal to k_{off} for the wild-type enzyme, was measured to be 0.3 s^{-1}. The results suggest that, at least in the case of the R283K mutant, the enzyme's affinity for the DNA substrate is not reduced relative to wild type.

Though R283A and R283K were much less efficient catalysts, the fidelity of DNA synthesis, in terms of the T-G mispair *vs* the T-A correct pair, achieved by these mutants was reduced only by a factor of 2 relative to that of wild-type pol β in the presence of magnesium (Table 2). The only significant decrease in fidelity was observed with R283A in the presence of manganese. The results suggest that the hydrogen bond between R283 and the template base plays a role in discriminating between the T-A and T-G base pairs, but it is only one of many factors that contribute to the nucleotide specificity of the active site.

The Putative Conformational Change Step. As shown in Scheme 1, the catalysis by pol β could involve a conformational change (step 3) prior to the chemical step (step 4); the k_{pol} could represent the combined rate constants k_3 and k_4. Direct observation of the conformational step awaits further structural studies. However, indirect evidence for the existence of the conformational change step was obtained by showing that the chemical step is only partially rate-limiting in the pre-steady-state catalysis.

If the chemical step in the reaction contributes to rate limitation, then k_{pol} should decrease when dNTPαS is used as a substrate analog of dNTP; *i.e.*, the "thio effect", defined as $k_{pol(dNTP)}/k_{pol(dNTP\alpha S)}$, should be greater than 1. The thio effects under various conditions are listed in Table 4. Upon substitution of dATP with dATPαS, the thio effect is 4.3. The thio effect for model S_N2 reactions involving a phosphodiester electrophile, 2,4-dinitrophenyl phosphate, and various nucleophiles ranges from 4−11 (Herschlag *et al.*, 1991). The small thio effect observed in the reaction catalyzed by pol β suggests that the chemical step is only partially rate-limiting and implies the existence of another step, most likely a conformational change, that also contributes to k_{pol}.

The notion that the chemical step is only partially rate-limiting is consistent with the observation that the thio effect of WT increases to 9 for incorporation of the incorrect nucleotide dGTP; i.e., the chemical step became more rate-limiting during incorrect dNTP incorporation.

Site-specific mutants could also be used, in complementation to the use of incorrect nucleotides, to raise the activation energy of the chemical step and thus make the chemical step more rate-limiting. Thus one might expect to see a larger thio effect on the reactions catalyzed by R283K and R283A than by wild-type pol β if the chemical step was the major rate-limiting factor in catalysis by these mutants. However, the thio effects observed for the mutant enzymes, 3.1 for R283K and 2.8 for R283A, were similar to that of WT. The thio effects for incorrect, dGTP, incorporation were also similar to that of wild type, as shown in Table 4. Possible explanations are provided in the Discussion.

DISCUSSION

Pol β shares more sequence homology with terminal nucleotidyl transferases (Holm & Sander, 1995) than with T4 pol, T7 pol, KF, or HIV RT (Delarue *et al.*, 1990). However, the results of the first pre-steady-state analysis of pol β indicate that the catalytic properties of pol β are similar to those of the enzymes mentioned and suggest that pol β could be an excellent model for further studies of the detailed structure−function relationship of polymerases catalyzing template-directed nucleotidyl transfer reactions.

The Processivity of Pol β Is Poor. Our results indicate that pol β can catalyze DNA synthesis processively as described in Scheme 1. Processive catalysis by pol β was observed previously on short gapped substrates but not on single-stranded substrates (Singhal & Wilson, 1993; Prasad *et al.*, 1994). As shown in Table 1, the processivity of pol β is low compared to that of other polymerases but is comparable to that of T4 pol *in vitro*. *In vivo*, the processivity of T4 pol holoenzyme reported in Table 1 is much greater than the processivity of isolated T4 pol (Capson *et al.*, 1992), presumably due to the presence of accessory proteins that can enhance the affinity of the polymerase for DNA and thus reduce k_{off}. Thus, pol β could be more processive *in vivo* if accessory proteins associate with it to cause an increase in k_{pol} or a decrease in k_{off} for the pol β·-DNA complex.

The Fidelity of Pol β Is Low. Pol β has been estimated previously to have the lowest fidelity of all eukaryotic DNA polymerases by forward mutation assay (Kunkel & Alexander, 1986). Of the 12 possible mispairs, 4 (T:G, G:T, A:C, and C:A) that can hydrogen bond to closely resemble Watson−Crick-type base pairs (Patel *et al.*, 1982; Gao & Patel, 1987; Brown *et al.*, 1985; Hunter *et al.*, 1986) were made most often. The fidelity of DNA synthesis, in terms of the T-A *vs* T-G base pairs, achieved by pol β is compared with the fidelities of other polymerases measured by kinetics in Table 5. The fidelity of DNA synthesis is dependent on the sequence of the DNA template as well as the mispair. The 25/45mer used in this study was identical to the one used in the study of HIV RT (Kati *et al.*, 1992) so that the fidelity of these enzymes could be compared directly. As shown in Table 5, the fidelity of pol β is similar to that of

Table 5: Comparison of the Fidelity of Pol β, T7 DNA Polymerase, Klenow Fragment, and HIV Reverse Transcriptase As Determined by Pre-Steady-State Kinetics

enzyme	dNMP:dNTP	k_{pol}/K_d (M^{-1} s^{-1})	fidelity
pol β[a]	T:A	9.1×10^4	
pol β	T:G	150	610
KF[b]	T:A	1.0×10^7	
KF(exo$^-$)	T:G	520	1.9×10^4
T7(exo$^-$)[c]	T:A	1.5×10^7	
T7(exo$^-$)	T:G	30	5.0×10^5
HIV RT[d]	T:A	8.2×10^6	
HIV RT	T:G	4.8×10^3	1.7×10^3

[a] The concentration of dNTP that gives the half-maximal rate of catalysis, K_d^{app}, was used for pol β. [b] Carroll *et al.* (1991). [c] Donlin and Johnson (1994). [d] Kati *et al.* (1992).

HIV RT, which is notorious for its infidelity. This result is consistent with the conclusion by Kunkel and Alexander (1986) that the frequency of misincorporation by pol β is the highest of all eukaryotic DNA polymerases on the basis of the forward mutation assay. There is a possibility that pol β is actually less faithful than it appears to be due to misalignment−mispairing (Kunkel, 1992) of the primer-template substrate, but this factor should not have a significant impact on our interpretations since we are mainly comparing pol β with other polymerases or with mutants of pol β.

The affinity of the active site for the correct nucleotide, dATP, was 8 times greater than the affinity for the incorrect nucleotide, dGTP, as measured by K_d^{app} in the presence of magnesium. The results suggest that the incorrect nucleotides are initially selected against in the ground state. An incorrect base pair, T-G, is also selected against by a slow rate of incorporation (Table 2). The slow rate of incorporation could be due to the enzyme's relative inability to stabilize the transition state of the chemical step involving the incorrect nucleotide or to a decrease in the rate of a conformational change after binding of the incorrect nucleotide that could bring catalytic residues into proper position for catalysis (Wong *et al.*, 1991).

The Thio Effect and Rate-Limiting Step(s). The factor of 4.3 decrease in catalytic rate observed when S_p-dATPαS was substituted for dATP is small but in the range, 4−11, expected for model S_N2 reactions (Herschlag *et al.*, 1991). The result suggests that the chemical step is only partially rate-limiting in the processive catalysis of DNA synthesis by pol β. Another plausible rate-limiting step could be a dNTP-induced conformational change (step 3 in Scheme 1), because (i) the thio effect of pol β is comparable to that of KF, 3−7 in the steady state (Kuchta *et al.*, 1987), T7 pol, 3.1 (Patel *et al.*, 1991), and T4 pol, 2 (Capson *et al.*, 1992), and (ii) there is good evidence for rate-limiting conformational changes prior to nucleotidyl transfer in the mechanisms of KF and T4 pol (Frey *et al.*, 1995) as well as T7 pol (Wong *et al.*, 1991). Fluorescent studies are in progress to characterize the putative conformational change step.

The thio effect for incorrect incorporation by WT pol β (9) is smaller than the thio effect for incorrect incorporation catalyzed by KF (32 in the presence of manganese) and T7 pol (60) (Eger *et al.*, 1991; Wong *et al.*, 1991). It is unclear why the thio effects for incorrect nucleotides differ greatly between different polymerases and why some of them exceed the value of 4−11 suggested by Herschlag *et al.* (1991). We believe the intrinsic thio effect varies from enzyme to enzyme, depending on the detailed mechanism. The maximal thio effect observed thus far for pol β is 11, as shown in Table 4.

The partially rate-limiting conformational change can also be involved in controlling the fidelity of polymerases. It has been proposed that T7 DNA pol selects against mispairs in an initial binding step in which the incorrect nucleotide binds weakly and in a second step in which a conformational change, which brings catalytic factors into optimal positions, occurs much more rapidly upon formation of a Watson−Crick-type base pair than upon formation of a mispair (Wong *et al.*, 1991).

Structural and Functional Roles of Arg-283. The similarity between the 2D NMR spectra of WT and the R283A, R283K mutants suggested that the structure of the mutants is not perturbed in the free enzyme form. This was further supported by the lack of change in the conformational stability of the mutants. The results of the structural analyses indicate that Arg-283 does not play a structural role and establish the basis for interpreting the functional data of the mutants.

The results presented in Table 2, demonstrating that R283 is important for catalysis, lend credibility to the catalytic relevance of the pseudoternary pol β crystal structure (Pelletier *et al.*, 1994). In this structure that closely approximates the ground state, R283 hydrogen bonds directly with the nitrogenous base of the template that forms a Watson−Crick base pair with the incoming dNTP (Figure 1). If the hydrogen bond is maintained as the enzyme−substrate complex enters the transition state, then it should be critical for positioning the base pair so that the α-phosphoryl group of the dNTP substrate is in optimal position for nucleophilic attack by the 3′-OH group of the primer near the catalytic aspartate residues.

If only the rate of the chemical step was reduced upon mutation of R283, then one would expect that the thio effect for the mutants to be greater than for WT pol β. However, the thio effect did not increase (decreased slightly) upon mutation of R283. The results suggest that the mutation causes an almost equivalent decrease in the rate of the chemical step and the rate of at least one other rate-limiting step, possibly a dNTP-induced conformational change. If only the rate of a conformational change was slowed, then one would expect the thio effect to decrease. Thus, the results suggest that mutations of Arg-283 could perturb the putative conformational step (step 3) and the chemical step (step 4) evenly. Consistent with this interpretation, the thio effects of the mutants for the incorporation of incorrect nucleotides (9 and 11 for R283A and R283K, respectively) are also similar to that of WT (9).

It is surprising that the mutation of a residue (R283) that hydrogen bonds directly with the base pair and is important for catalysis does not have a greater effect on fidelity, in terms of the T-G mispair *vs* the T-A correct pair. The results suggest that the hydrogen bond between R283 and the template base is only one of several factors that contribute to fidelity. While 3′ → 5′ exonuclease and DNA mismatch repair contribute to the fidelity of DNA replication in the cell, the specificity of the polymerase active site for correct base pairs is the most significant contributing factor to fidelity. R283 is one of many residues, including D190, D192, D256, Y271, F272, D276, N279, and L287, that form the binding pocket of the base pair at the active site of pol

β. Mutations at position 283, in conjunction with mutations at the other residues in the binding pocket, should have a much greater effect on the fidelity than the single point mutation.

It is also surprising that mutations at position R283 did not have a greater effect on the affinity of the enzyme for the DNA substrate because R283 hydrogen bonds directly with the template. It is possible that more than one of the residues that collectively interact with the DNA substrate will have to be changed before significant effects on the enzyme's affinity for DNA are observed.

Conclusion. We present the first pre-steady-state analysis of the reaction catalyzed by pol β and detailed structure−function analyses of R283A and R283K mutants. The fidelity of pol β, in terms of the T-G mispair *vs* the T-A correct pair, is poor. The enzyme makes as many mistakes as HIV RT, which is notorious for its low fidelity. The processivity of pol β is also lower than that of KF and HIV RT. Arg-283 is important for the catalytic efficiency of pol β but not the structure. The results of the kinetic studies suggest that the small and simple pol β is a good model for template-directed nucleotidyl transfer, sharing many characteristics, including a possible substrate-induced conformational change, with more complicated polymerases such as KF, T7 pol, T4 pol, and HIV RT.

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