DNA Polymerase β: Structure−Fidelity Relationship from Pre-Steady-State Kinetic Analyses of All Possible Correct and Incorrect Base Pairs for Wild Type and R283A Mutant†

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ABSTRACT: The kinetic parameters (k_{pol}, K_d^{app}) for all possible correct and incorrect pairing between the A, T, G, and C bases were determined for wild-type (WT) rat DNA polymerase β (pol β) and the R283A mutant under pre-steady-state kinetic assay conditions. The base substitution fidelities of these two proteins were then determined for all 12 possible mispairs representing the first complete fidelity analysis of polymerases using pre-steady-state kinetics. The results led to several significant findings: (i) For both WT and R283A, the fidelity is determined primarily by k_{pol} (decreases for the incorporation of incorrect nucleotides) and to a small extent by K_d^{app} (increases for the incorporation of incorrect nucleotides). (ii) In general, the fidelity for the Y·X (incorporation of dXTP opposite template dYMP) mismatch is different from that for the X·Y mismatch, reflecting the asymmetry of the active site. (iii) The fidelity of R283A is reduced in all 12 mispairs compared to that of WT. The extent of decrease varies from 200-fold for the A·G mispair to 2.5-fold for the T·C mispair. In general, the differences in fidelity between the mutant and WT are greater for purine·purine mismatches (up to 200-fold) than purine·pyrimidine, pyrimidine·purine, or pyrimidine·pyrimidine mismatches (up to 19-fold). (iv) Overall, the decreases in the fidelity of the R283A mutant are caused mainly by changes in the values of k_{pol}; the k_{pol} values of correct incorporations decrease to a greater extent for the R283A mutant with respect to WT than those of incorrect incorporations. With the exception of G·C, the values of K_d^{app} for the WT and R283A mutant remain constant for correct pairings and vary by less than a factor of 4 for incorrect pairings. (v) For WT pol β, the K_d^{app} of G·C (8.6 μM) is distinctly smaller than that of other correct base pairs (41−108 μM). For the R283A mutant, the k_{pol} of G·C is higher by a factor of 15−17.

Rat DNA polymerase β (pol β)[1] is one of the smallest (39 kDa) 5′ → 3′ DNA polymerases known. It is also one of the simplest DNA polymerases since it lacks other intrinsic activities such as 3′ → 5′ or 5′ → 3′ exonuclease activity or endonuclease activity (Tanabe *et al.*, 1979). The enzyme consists of two domains (Kumar *et al.*, 1990): a 31 kDa domain consisting of the catalytic center for DNA polymerization, and an 8 kDa domain involved in releasing the 5′-terminal deoxyribose phosphate residues from incised apurinic·apyrimidinic sites as a part of the base excision repair process (Matsumoto & Kim, 1995). This mammalian pol β has been cloned and overexpressed in *Escherichia coli* (Zmudzka *et al.*, 1986; Matsukage *et al.*, 1987). The recombinant enzyme has been purified to homogeneity and found to be fully active compared to the enzyme purified from the original sources (Date *et al.*, 1988; Abbotts *et al.*, 1988). The crystal structure of the enzyme in the ternary complex form with DNA template-primer and ddCTP has been solved at a resolution of 2.9 Å (Pelletier *et al.*, 1994). The relative simplicity of the system and the available crystal structure of the ternary complex form present an opportunity to study its structure−fidelity relationship in great depth.

Recently we first reported the use of pre-steady-state kinetics to determine the k_{pol}, K_d^{app}, and fidelity of WT pol β and Arg-283 mutants in the misincorporation of dGTP opposite template dTMP (Werneburg *et al.*, 1996). According to the crystal structure (Pelletier *et al.*, 1994), Arg-283 forms a hydrogen bond with the base of the template that hydrogen bonds with the incoming dNTP substrate as shown in Figure 1. After demonstrating that the structures of the mutants were not globally perturbed, the kinetic data allowed us to conclude that Arg-283 is important for catalysis and fidelity.

In order to fully understand the functional role of R283 in substrate specificity and fidelity, we have extended our kinetic characterizations to all 4 possible correct and 12 possible incorrect base pair formations between the A, T, G, and C bases. Such a complete analysis of fidelity by the

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[1] Abbreviations: 25/45mer, 25mer oligonucleotide annealed to a 45mer oligonucleotide as described under Materials and Methods; BSA, bovine serum albumin; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; dNMP, 2′-deoxynucleoside 5′-monophosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HIV RT, human immunodeficiency virus reverse transcriptase; KF, Klenow fragment of DNA polymerase I; KF(exo⁻), a site-specific mutant of KF with diminished 3′ → 5′ exonuclease activity; pol β, rat DNA polymerase β; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; T4 pol, T4 DNA polymerase; T7 pol (exo⁻), a site-specific mutant of T7 DNA polymerase with diminished 3′ → 5′ exonuclease activity; TLC, thin-layer chromatography; Tris·HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; WT, wild type.

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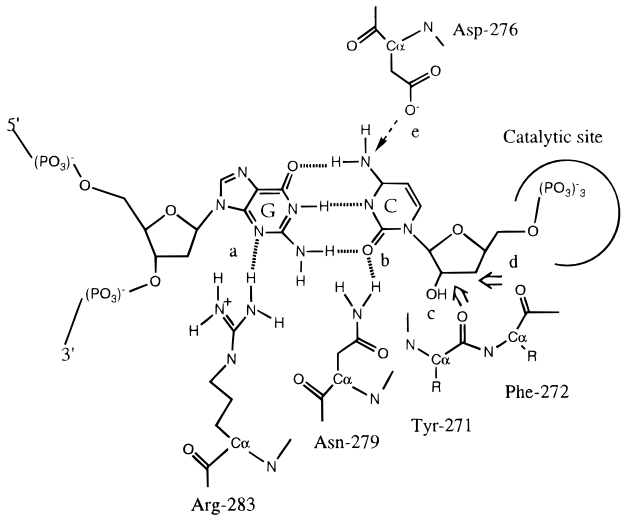

FIGURE 1: Schematic representation of the incoming dNTP binding pocket formed by the template base and active site residues based on the X-ray crystal structure of the pol β·DNA·ddCTP complex (Pelletier *et al.*, 1994). It was proposed that the side chains of Arg-283 and Asn-279 have hydrogen bonding interactions with DNA bases (sites a and b, respectively), and the backbone carbonyls of Tyr-271 and Phe-272 (c and d, respectively) form the binding pocket for the 2′-deoxyribose ring of the incoming dNTP. The carboxylate group of Asp-276 is in a close distance with the N-3 of dCTP (4.8 Å, e).

pre-steady-state kinetics has never been performed for any other polymerase. The data allowed us to address several issues relevant to the structure−function relationship of pol β, including the dNTP specificity, the "symmetry problem" (whether the Y·X pair behaves the same as the X·Y pair, where Y·X represents incorporation of dXTP opposite template dYMP), the structure−fidelity relationship, and the specific roles of Arg-283 in catalysis and fidelity. The work represents a step forward in the analysis of the structure−function relationship of pol β in particular and DNA polymerases in general.

MATERIALS AND METHODS

Materials. Ultra-pure dNTPs were purchased from Pharmacia, and [γ-^{32}P]ATP and [α-^{32}P]dNTP were from ICN Biomedicals. Nuclease and protease activity free BSA was obtained from Boehringer Mannheim. T4 polynucleotide kinase was purchased from New England Biolabs. Sequenase 2.0 was purchased from United States Biochemical. Centricon-3 was purchased from Amicon (Beverly, MA), and Sep-Pak C18 cartridges were obtained from Waters (Milford, MA). DE-81 filters were obtained from Whatman. G-25 microspin columns were purchased from Pharmacia Biotech. All other reagents were of the highest purity available commercially. WT pol β and the R283A mutant were purified as described (Date *et al.*, 1988) from an overexpressing *Escherichia coli* system, BL21(DE3)[plysS, pET17-polβ] (Werneburg *et al.*, 1996). The enzymes were estimated to be greater than 98% homogeneous based on SDS−PAGE analysis developed by the silver staining method (Poehling & Neuhoff, 1981). The exact concentration of the enzyme solution was determined by using an extinction coefficient at 280 nm (21 200 M^{-1} cm^{-1}) of WT pol β (Casas-Finet *et al.*, 1991). It was assumed that the mutation at R283 did not change the extinction coefficient.

DNA Substrates. Custom-synthesized DNA oligomers were purchased from IDT Inc. (Coralville, IA). The 25mer and 45mer were purified by electrophoresis on 16% acrylamide−7 M urea gels. The correct sizes of DNA oligomers as major bands were identified by UV shadowing with TLC plates. The portions of gels containing 25mer and 45mer were homogenized by passing them through 10 mL syringes. They were eluted with 0.1 M triethylammonium acetate, 1 mM EDTA, pH 7.0, at 37 °C with shaking (∼250 rpm) overnight. The solutions were then desalted on Sep-Pak C18 cartridges. The DNA duplex, 25/45mer, was formed by heating the mixture at 85 °C for 15 min and slowly cooled down to room temperature. The DNA duplex was purified with 10% acrylamide gels, eluted as described above, and buffer-exchanged with 10 mM Tris, 1 mM EDTA, pH 8.0, by using Centricon-3.

Four different DNA duplexes were used for 4 correct and 12 incorrect dNTP incorporations. The sequences of these DNA duplexes are as follows:

25A/45TT

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5′GCCTCGCAGCCGTCCAACCAACTCA
3′CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG5′
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25A/45TA

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5′GCCTCGCAGCCGTCCAACCAACTCA
3′CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG5′
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25A/45TC

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5′GCCTCGCAGCCGTCCAACCAACTCA
3′CGGAGCGTCGGCAGGTTGGTTGAGTCGGAGCTAGGTTACGGCAGG5′
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25A/45TG

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5′GCCTCGCAGCCGTCCAACCAACTCA
3′CGGAGCGTCGGCAGGTTGGTTGAGTGCGAGCTAGGTTACGGCAGG5′
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where the name of each duplex is given as the length of primer/template in combination with the DNA base letters for the next available template base and the previous base pair. For example, 25A/45TC indicates the correct dNTP for incorporation is dGTP and the previous base pair is T·A.

The exact concentration of the DNA duplex was determined by incorporation of the correct [α-^{32}P]dNTP by using Sequenase 2.0. In a total volume of 75 μL were incubated 20 nM 25/45mer, 1 μM appropriate [α-^{32}P]dNTP (25 Ci/mmol), 2 unit of Sequenase 2.0, 20 mM $MgCl_2$, 50 mM NaCl, and 40 mM Tris·HCl, pH 7.5, at 25 °C. After 1−3 min, 20 μL aliquots were withdrawn and quenched with 10 μL of 0.5 M EDTA, pH 8.0. The amount of radioactivity incorporated into DNA substrate was determined by the DE81 filter binding assay (Lindell *et al*., 1970; Kuchta *et al*, 1987). The radioactivity was the same for three different reaction times. The background level of radioactivity was detected when the correct [α-^{32}P]dNTP was replaced with incorrect [α-^{32}P]dNTP in the reaction. These acertain that all of the primer was extended, and the misincorporation of [α-^{32}P]dNMP was not accounted in the determination of 25/45mer concentration.

The DNA duplex (25/45mer) was 5′-end-labeled with ^{32}P by incubating with T4 polynucleotide kinase and [γ-^{32}P]ATP (4500 Ci/mmol) following the manufacturer's protocol. The 5′-radiolabeled 25/45mer was separated from unreacted [γ-^{32}P]ATP with a G-25 microspin column. The labeled DNA was mixed with *ca.*100-fold molar excess of unlabeled

25/45mer, and the T4 polynucleotide kinase was inactivated at 65 °C for 15 min. The solution was slowly cooled down to room temperature. When the substrate was prepared in this way, the degree of conversion to the 26/45mer was greater than 95%.

Determination of the k_{pol} and K_d^{app} for dNTP. The rate of incorporation of the correct base onto the 25/45mer duplex was determined in a reaction containing a final concentration of 50 nM 25/45mer (5′-radiolabeled), 12.5−300 μM appropriate dNTP (5−300 μM for dCTP due to the lower K_d^{app} for WT), and 500 nM pol β in 50 mM Tris·HCl, pH 7.6 at 37 °C, 2.5 mM $MgCl_2$ (as a free form), 0.1 mM EDTA, 50 mM KCl, and 0.2 mg/mL BSA. The reaction was initiated by combining the solution containing the enzyme and DNA substrate with the second solution containing Mg^{2+}/dNTP. The reaction was quenched with 0.25 M EDTA (final concentration), pH 8.0. A rapid quench instrument (KinTek Instrument Corp., State College, PA) was used for reaction times ranging from 20 ms to 20 s. For reaction times greater than 20 s, 20 μL aliquots of the reaction mixture (total volume 140 μL) were removed and mixed with equal volumes of 0.5 M EDTA, pH 8.0, manually with a certain time interval. A sample of the quenched reaction mixture (20 μL) was mixed with an equal volume of the gel loading buffer (Maniatis *et al.*, 1982), denatured at 85 °C for 5 min, and loaded onto a 16% acrylamide−7 M urea gel. The substrate and product bands were quantitated with a β-scanner (Betagen) as described by Werneburg *et al*. (1996).

The rate of misincorporation for a given mismatch was determined by mixing a solution containing enzyme and DNA (as described above) with 100−1500 μM of the appropriate dNTP for the reaction time ranging from 20 to 3600 s. For the reaction time ranging from 1 to 20 s, the rapid quench instrument was used. The data were collected as described above.

Exonuclease Activity. Purified enzymes were tested for the exonuclease activity of contaminating *E. coli* DNA polymerase I. The enzyme, 1 μM, was incubated with 0.4 μM of 5′-radiolabeled DNA substrate ([5′-α-^{32}P]25A/45TT), 1 mM $MgCl_2$, 1 mM DTT, and 0.4 mg of BSA for 3 h at 25 °C. There was no detectable decrease in [5′-α-^{32}P]25A when the reaction was run on a 16% acrylamide−7 M urea gel and quantitated with the β-scanner.

RESULTS

Determination of the k_{pol} and K_d^{app} for dNTP. The minimal kinetic mechanism for pol β can be represented as follows:

$$E \underset{k_{off}}{\overset{k_{on}[D_n]}{\rightleftharpoons}} ED_n \underset{k_{-2}}{\overset{k_2[N]}{\rightleftharpoons}} ED_nN \xrightarrow{k_{pol}} ED_{n+1}P \qquad (1)$$

where E, D_n, N, and P represent pol β, primer/template DNA, dNTP, and inorganic pyrophosphate, respectively, and k_{pol} is the catalytic turnover number. It has been established that the DNA duplex binds to pol β before dNTP in an ordered fashion (Tanabe *et al*., 1979). The concentration of dNTP that gives the half-maximal rate of catalysis was defined as K_d^{app} (Werneburg et al., 1996).

Pre-steady-state kinetic experiments were performed by monitoring the formation of 26mer product from a 25mer substrate (in the form of 25/45mer DNA duplex) in the presence of a single dNTP. The data were fit to a single exponential curve (Carroll et al., 1991) to obtain the catalytic rate observed, k_{obs}, at various concentrations of dNTP:

$$[26mer] = A[1 - \exp(-k_{obs}t)] \qquad (2)$$

where A is the initial concentration of DNA duplex substrate. Examples of the product versus time plots for WT and R283A are shown in Figures 2A and 3A, respectively.

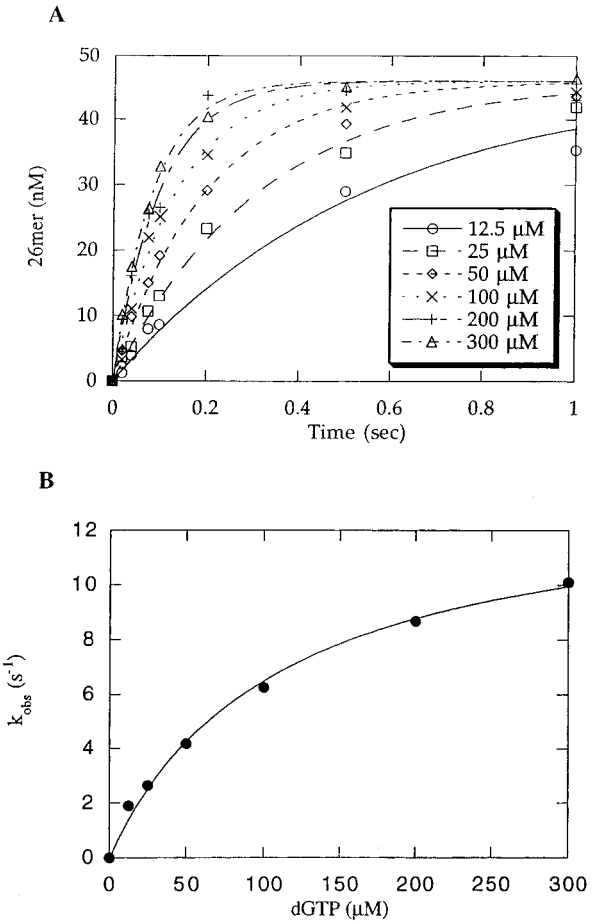

FIGURE 2: Pre-steady-state kinetics of dGTP incorporation into the 25A/45TC catalyzed by WT. (A) Product *vs* time plot. The data were obtained as described under Materials and Methods and fit with a single exponential curve (eq 2) to obtain the k_{obs} for each dGTP concentration. (B) dGTP concentration dependence of the catalytic rate observed. The k_{pol} and K_d^{app} were estimated to be 13.5 ± 0.7 s^{-1} and 108 ± 13 μM, respectively, by fitting k_{obs} vs [dGTP] with the hyperbolic equation (eq 4).

A 10-fold excess concentration of the enzyme relative to the DNA substrate was used for all assays. Under this condition, >90% of the DNA substrate should be complexed to the enzyme on the basis of the K_d of DNA (25A/45TT) bound to WT pol β determined previously (Werneburg *et al.*, 1996). This was further confirmed by the observation that 5-fold and 10-fold excess of enzyme concentrations over 25A/45TT gave the same catalytic rate (k_{obs}) at 100 μM dATP for both WT and R283A.

In some cases of misincorporation, for example, dATP into 25A/45TC, multiple misincorporation was observed at high concentration of dATP. In these cases, the decrease of substrate (25mer) was fit to the single exponential equation (eq 3) to obtain the rate of polymerization at each dATP concentration:

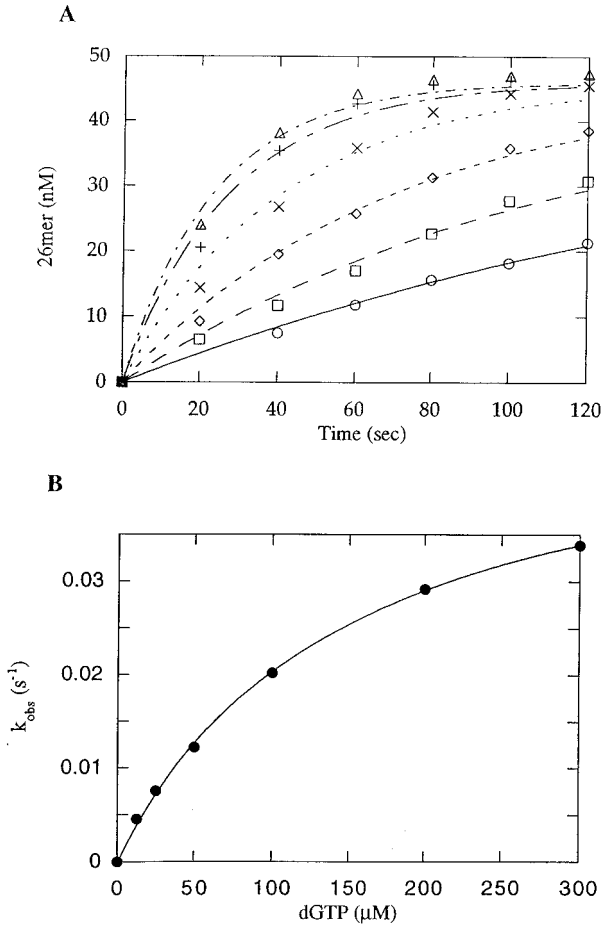

FIGURE 3: Pre-steady-state kinetics of dGTP incorporation into the 25A/45TC catalyzed by R283A. (A) Product *vs* time plot. The data were obtained as described under Materials and Methods and fit as described in Figure 2A for each dGTP concentration. The concentrations of dGTP were the same as in Figure 2A. (B) dGTP concentration dependence of the catalytic rate observed. The data were fit as described in Figure 2B. The k_{pol} and K_d^{app} were estimated to be 0.051 ± 0.001 s^{-1} and 151 ± 9 μM, respectively.

$$[25\text{mer}] = A \exp(-k_{obs}t) \quad (3)$$

It was assumed that dNTP binding and dissociation are in rapid equilibrium. This assumption is supported by the experimental observation that the polymerization reaction can be fit with a single exponential equation (eq 2 or 3) at all concentrations of dNTP used (Patel *et al*., 1991). If the dNTP binding step is not reversible and rapid, the polymerization reaction would have a lag phase at low concentrations of dNTP (Johnson, 1995).

The observed catalytic rates (k_{obs}) were then plotted against the dNTP concentrations used, and the data were fit with the hyperbolic equation (eq 4) to determine the k_{pol} and K_d^{app}:

$$k_{obs} = k_{pol}[\text{dNTP}]/([\text{dNTP}] + K_d^{app}) \quad (4)$$

Figures 2B and 3B show examples of such plots for WT and R283A, respectively. The resulting values of k_{pol} and K_d^{app} for both WT and R283A, along with the ratio k_{pol}/K_d^{app} and the fidelity (to be addressed in a later section) for all possible correct and incorrect incorporations, are summarized in Tables 1−4.

As shown in Tables 1−4, the k_{pol} values of all four correct base pairs are significantly reduced for the R283A mutant. It is important to determine if the reduction in k_{pol} is caused by a reduction in the affinity for the DNA duplex, since the side chain of R283 forms a hydrogen bond with the template base as shown in Figure 1. The dissociation constant (K_d) for DNA binding to the WT pol β has been determined as 49 ± 12 nM previously by titrating the burst amplitude with the 25/45mer DNA (Werneburg *et al*., 1996). This method, however, is not applicable to R283A due to the low catalytic rate and thus the lack of burst. As an alternative, we used steady-state kinetics to determine the K_m of DNA for the R283A mutant. The result is shown in Figure 4. The K_m value thus obtained, 46 ± 6 nM, is similar to the K_d value determined for WT. This result, coupled with the fact that 10-fold excess of enzyme over DNA was used in all assays, assures that DNA binding affinity does not play a role in the differences in the k_{pol} values between WT and R283A.

Substrate Specificity toward Correct dNTP. The rate of correct nucleotide incorporation varies from 9.4 s^{-1} for the incorporation of dCTP opposite template dGMP (G·C) to 24.1 s^{-1} for the T·A base pair for WT. It is surprising to observe that the K_d^{app} of dCTP for the G·C pairing is only 8.6 μM, while that of other dNTP for correct incorporation varies from 41 to 108 μM. The T7 pol (exo$^-$) is the only other polymerase for which the values of K_d for all four correct dNTP have been reported (Donlin & Johnson, 1994); for this enzyme, the K_d is essentially the same for all four dNTPs. Upon conversion of Arg-283 to Ala, this distinguishing tight binding of dCTP by pol β disappears. The K_d^{app} of G·C is comparable to or higher than that of other correct pairs for R283A.

Substrate Specificity toward Incorrect dNTP. In general, the rate of misincorporation of dNTPs onto a template base varies in a wider range with WT than with R283A. As an example, Figure 5 shows the plots of the rates of misincorporation for three different mismatches, dATP, dTTP, and dGTP, into 25A/45TG for WT (Figure 5A) and R283A (Figure 5B). The k_{pol} of WT varies in a 15-fold range, from 0.11 to 0.0073 s^{-1}, while the rate of R283A varies in only a 2-fold range, from 0.016 to 0.0091 s^{-1}. The K_d^{app} for both enzymes varies by less than 2-fold. The highest K_d^{app} for WT is 380 μM, and the lowest K_d^{app} is 210 μM. The corresponding values for R283A are 700 and 450 μM, respectively.

As shown in Tables 1−4, WT and R283A also display different substrate specificities for misincorporation. The "most favorable mispairing" for WT is T·C or G·T ($k_{pol}/K_d^{app} = 290$), while the most favorable mispairing for R283A is G·A ($k_{pol}/K_d^{app} = 33$). The least favorable misincorporation for WT is A·G ($k_{pol}/K_d^{app} = 8.1$); that of R283A is C·T ($k_{pol}/K_d^{app} = 0.09$).

Fidelity. The fidelities of 12 different base misincorporations for WT and R283A were calculated from the k_{pol} and K_d^{app} data according to eq 5:

$$\text{fidelity} = [(k_{pol}/K_d^{app})_c + (k_{pol}/K_d^{app})_i]/(k_{pol}/K_d^{app})_i \quad (5)$$

where c and i represent correct and incorrect incorporations, respectively. The fidelity data thus obtained are also listed in Tables 1−4. Four DNA duplexes (25/45mer) were used for this purpose. It has been shown that pol β and other polymerases such as *E. coli* KF and mammalian DNA

Table 1: Base Substitution Fidelity of WT and R283A on 25A/45TA

		A·T[a]	A·C	A·G	A·A
WT	k_{pol} (s^{-1})	16.7 ± 0.7	0.020 ± 0.001	0.0079 ± 0.0010	0.0060 ± 0.0003
	K_d^{app} (μM)	41 ± 6	200 ± 35	980 ± 200	290 ± 50
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	410000	100	8.1	21
	fidelity	N/A	4100	51000	20000
R283A	k_{pol} (s^{-1})	0.054 ± 0.005	0.00039 ± 0.00010	0.00120 ± 0.00003	0.00032 ± 0.00010
	K_d^{app} (μM)	61 ± 17	530 ± 140	350 ± 30	1250 ± 690
	k_{pol}/K_d^{app} (M^{-1s-1})	890	0.74	3.4	0.26
	fidelity	N/A	1200	260	3400
WT/R283A[b]		N/A	3.4	200	5.9

[a] The correct base pair and mispair are represented as dNMP·dNTP. [b] The ratio of fidelity of WT/R283A.

Table 2: Base Substitution Fidelity of WT and R283A on 25A/45TT

		T·A[a]	T·G	T·C	T·T
WT	k_{pol} (s^{-1})	24.1 ± 0.9[c]	0.13 ± 0.02[d]	0.18 ± 0.01	0.0085 ± 0.0004
	K_d^{app} (μM)	52 ± 6[c]	850 ± 320[d]	630 ± 60	820 ± 80
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	460000	150	290	10
	fidelity	N/A	3100	1600	46000
R283A	k_{pol} (s^{-1})	0.048 ± 0.004[d]	0.0014 ± 0.0001[d]	0.00096 ± 0.00004	0.00012 ± 0.00002
	K_d^{app} (μM)	64 ± 13[d]	575 ± 11[d]	800 ± 60	1100 ± 290
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	750	2.4	1.2	0.11
	fidelity	N/A	310	630	6800
WT/R283A[b]		N/A	10	2.5	6.8

[a] The correct base pair and mispair are represented as dNMP·dNTP. [b] The ratio of fidelity of WT/R283A. [c] The values of k_{pol} and K_d^{app} are slightly different from the previous results in Werneburg *et al.* (1996). Our current data are more accurate because the ratio of enzyme to the 25/45mer was 10:1 which is sufficient to saturate the 25/45mer with enzyme. The previous results were obtained with a 1:1 ratio of enzyme to the 25/45mer. [d] Werneburg *et al*. (1996), in the presence of a 5-fold excess of pol β over the 25/45mer.

Table 3: Base Substitution Fidelity of WT and R283A on 25A/45TG

		G·C[a]	G·T	G·A	G·G
WT	k_{pol} (s^{-1})	9.4 ± 0.5	0.11 ± 0.01	0.0162 ± 0.0004	0.0073 ± 0.0001
	K_d^{app} (μM)	8.6 ± 2.2	380 ± 80	210 ± 13	230 ± 10
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	1100000	290	77	32
	fidelity	N/A	3800	14000	34000
R283A	k_{pol} (s^{-1})	0.83 ± 0.08	0.016 ± 0.001	0.015 ± 0.002	0.0091 ± 0.0004
	K_d^{app} (μM)	170 ± 30	650 ± 80	450 ± 130	700 ± 60
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	4900	25	33	13
	fidelity	N/A	200	150	380
WT/R283A[b]		N/A	19	93	89

[a] The correct base pair and mispair are represented as dNMP·dNTP. [b] The ratio of fidelity of WT/R283A.

polymerases α and γ have a site preference for base substitution error frequency (Kunkel & Alexander, 1986; Bebenek *et al.*, 1990). Therefore, the four DNA duplexes used were designed to have the same sequences except one or two template bases at the site of incorporation.

The fidelity of WT pol β varies from 1600 for the misincorporation of dCTP into 25A/45TT to 51 000 for the misincorporation of dGTP into 25A/45TA, which gives a range of 32-fold. The fidelity of different mismatches increases in the following order: T·C, T·G, G·T, A·C, C·A, C·C, G·A, C·T, A·A, G·G, T·T, A·G. It is evident that fidelity is not symmetrical. For example, the fidelity of A·G is 51 000, and that of G·A is 14 000. The fidelities of A·C and C·A are similar. However, the substrate specificities of these two different mismatches are different, mainly due to a difference in the values of K_d^{app}. The asymmetry of substrate specificity for Y·X and X·Y was also observed with KF(exo$^-$) (Carroll *et al.*, 1991).

The fidelity of R283A varies from 150 for the misincorporation of dATP into 25A/45TG to 6500 for the misincorporation of dTTP into 25A/45TT, giving a range of 43-fold, which is comparable to (slightly larger than) that of the WT pol β. The fidelity of different mismatches increases in the following order, which is different from that of WT: G·A, G·T, A·G, T·G, G·G, T·C, C·C, A·C, C·A, A·A, C·T, T·T. The asymmetry of fidelity is still apparent for R283A, but to a lesser extent than WT.

Multiple Incorporation of Incorrect Bases. When the incorporation of an incorrect base into the DNA duplex was performed under the single-turnover reaction condition, multiple incorporation of the incorrect dNTP into DNA was observed in some cases. The catalytic turnover number, $k_{pol,2}$ for the second misincorporation was determined by using the mechanism as follows:

$$ED_n \xrightarrow{k_{pol,1}} ED_{n+1} \xrightarrow{k_{pol,2}} ED_{n+2} \quad (6)$$

Table 4: Base Substitution Fidelity of WT and R283A on 25A/45TC

		C·G[a]	C·A	C·T	C·C
WT	k_{pol} (s^{-1})	13.5 ± 0.7	0.025 ± 0.001	0.018 ± 0.002	0.012 ± 0.001
	K_d^{app} (μM)	108 ± 13	890 ± 70	2000 ± 380	580 ± 70
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	125000	28	9.0	21
	fidelity	N/A	4500	14000	6000
R283A	k_{pol} (s^{-1})	0.051 ± 0.001	0.00020 ± 0.00003	0.00012 ± 0.00002	0.00030 ± 0.00003
	K_d^{app} (μM)	151 ± 9	960 ± 370	1320 ± 220	1000 ± 170
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	340	0.21	0.09	0.30
	fidelity	N/A	1600	3700	1100
WT/R283A[b]		N/A	2.8	3.8	5.5

[a] The correct base pair and mispair are represented as dNMP·dNTP. [b] The ratio of fidelity of WT/R283A.

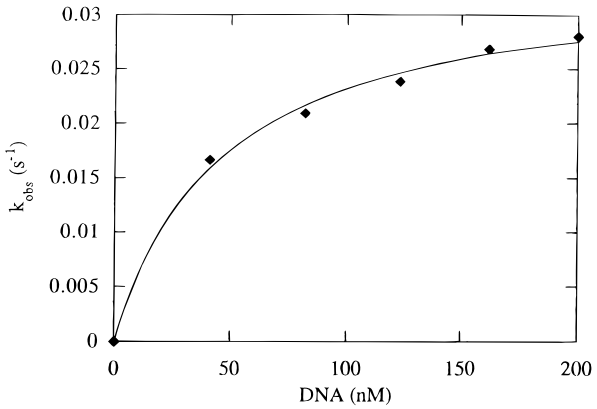

FIGURE 4: Steady-state rates of incorporation of dATP into 25A/45TT catalyzed by R283A, as a function of the DNA concentration. The initial rate was determined in a reaction containing a final concentration of 5 nM R283A, 150 μM dATP, and 40−200 nM 25/45mer (5'-radiolabeled) in the buffer as specified under Materials and Methods. The values of k_{cat} and K_m were estimated to be 0.034 ± 0.001 s^{-1} and 46 ± 6 nM, respectively, by fitting the data with the Michaelis−Menten equation.

The rates of formation of the first (D_{n+1}) and second (D_{n+2}) mismatches at various concentrations of dNTP were determined by using the simulation program Kinsim (Barshop *et al.*, 1983) as described in Carroll *et al.* (1991). The values of K_d^{app} were determined with the hyperbolic eq 4. Table 5 shows the kinetic constants of several multiple incorporations observed. In general, the k_{pol} for any type of the second misincorporation of dNTP is greatly reduced relative to the k_{pol} of the same type of the first misincorporation. For WT, it varies from a factor of 11 with G·A to a factor of 1250 with G·T. In the case of R283A, it changes from a factor of 24 with G·G to a factor of 65 with G·A. However, K_d^{app} remains essentially the same for both misincorporations. Interestingly, the second misincorporation of dTTP into 25A/45TT and 25A/45TC catalyzed by WT was not observed for R283A. This result suggests that formation of the first pyrimidine·pyrimidine mismatch could make the second misincorporation more difficult for R283A (relative to WT).

DISCUSSION

Specificity of WT and R283A toward Correct dNTPs. For the WT pol β, the values of k_{pol} for the incorporation of correct nucleotides are similar (9.4−24.1 s^{-1}). However, the K_d^{app} for dCTP incorporation opposite template dGMP (8.6 μM) is noticeably lower than that for the other correct dNTP incorporations (41−108 μM). There are two possible explanations for the lower K_d^{app} for G·C pair formation.

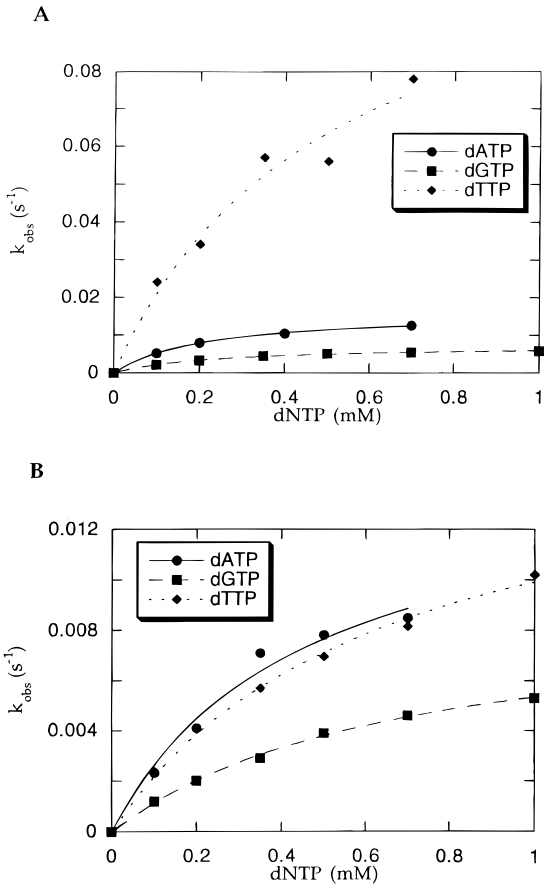

FIGURE 5: Rates of misincorporation of dATP, dGTP, and dTTP into 25A/45TG by WT pol β (A) and R283A (B). The assays were performed as described in Figure 2 and under Materials and Methods. The values of k_{pol} and K_d^{app} for dNTPs are listed in Table 3.

First, it could be the consequence of the slightly different template sequences used. The template base at the second site is G in the three templates used for the incorporations of dATP, dTTP, and dGTP. However, it cannot be a G for the incorporation of dCTP since otherwise the second correct incorporation will occur. Instead, C was used as shown in the sequence of 25A/45TG.

Alternatively, the relatively strong binding affinity of dCTP opposite template dGMP observed in WT could imply that pol β may have been optimized for G·U and/or G·T repairs. The G·C to A·T transition mutation accounts for one-third of single-site mutations observed in inherited human diseases (Cooper & Youssoufian, 1988). These types of mispairs are corrected by base excision repair involving pol β (Lindahl, 1993; Matsumoto & Kim, 1995). If the major biological function of pol β is to incorporate dCTP into a single gap,

Table 5: Multiple Misincorporation of dNTP into 25A/45TA, 25A/45TT, and 25A/45TC by WT and R283A

		AG·GG	AG·AA	TG·TT	CG·AA	CG·TT
WT	k_{pol} (s^{-1})	0.00060 ± 0.00010	0.00092 ± 0.00009	0.000088[b]	0.0015 ± 0.0001	0.0010 ± 0.0002
	K_d^{app} (μM)	560 ± 150	370 ± 110		550 ± 80	500 ± 230
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	1.1	2.5		2.7	2.0
R283A	k_{pol} (s^{-1})	0.00038 ± 0.00002	0.00036 ± 0.00012[b]	ND[c]	0.00023 ± 0.00003	ND[c]
	K_d^{app} (μM)	290 ± 40			670 ± 20	
	k_{pol}/K_d^{app} (M^{-1} s^{-1})	1.3			0.34	

[a] The mispairs are represented as template bases·primer bases. For example, the CG·TT indicates the first misincorporation base pair is the C·T and the second misincorporation base pair is the G·T. The kinetic parameters for the first misincorporation are shown in Tables 1, 2, and 4. The values of k_{pol} and K_d^{app} shown in this table are for the second misincorporation. [b] There were not enough data available to fit with a hyperbolic equation (eq 4). Thus, the second rate constant, k_{obs}, determined at 1.5 mM dNTP is shown. [c] There was no detectable accumulation of 27mer as a result of the second misincorporation.

it is reasonable for the enzyme to have lower K_d^{app} for dCTP compared to other correct dNTPs. It should be noted, however, that all of our experiments were performed with 25/45mers. The G·C specificity needs to be further examined by use of single-gapped DNA substrates before its biological relevance can be established.

The tight binding of dCTP was not observed in the case of R283A. However, the k_{pol} of dCTP incorporation increases by 15−17-fold compared with other correct dNTP incorporations. The ratio k_{pol}/K_d^{app} for G·C (4900 M^{-1} s^{-1}) remains higher than that for the other three correct base pairs (340−890 M^{-1} s^{-1}).

Fidelity of WT Pol β Is Determined Primarily by k_{pol}. The discrimination between correct and incorrect base pair formation in DNA polymerase synthesis can be achieved through two mechanisms in the absence of inherent 3′ → 5′ exonuclease: (1) tighter binding of correct nucleotides (relative to incorrect nucleotides) to the active site binding pocket formed by the enzyme and the DNA duplex substrate; (2) higher kinetic rates of incorporation of correct nucleotides (Goodman, 1988). The results described in this paper suggest that pol β selects correct nucleotide against incorrect nucleotides mainly through k_{pol}. In the case of WT, the ratio of the rate of correct to incorrect nucleotide incorporation ($k_{pol,c}/k_{pol,i}$) varies from 2800 for the selection of T·A against T·T to 85 for the selection of G·C against G·T. The ratio of the binding affinity for the correct nucleotide to that of incorrect nucleotides, $K_{d,i}^{app}/K_{d,c}^{app}$, varies in a substantially smaller range: from 44 for the selection of G·C against G·T to 5 for A·T against A·C. The selection against incorrect dNTPs by k_{pol} instead of K_d has also been observed with KF(exo⁻) (Carroll *et al.*, 1991). However, T7 pol (exo⁻), T4 pol, and HIV RT exhibit a great increase in the K_d for incorrect dNTPs (Wong *et al.*, 1991; Capson *et al.*, 1992; Kati *et al.*, 1992).

Fidelity of R283A Is Generally Lower than That of WT. As in the case for WT, the selection of correct against incorrect nucleotides mainly comes from the slower rate of misincorporation of dNTP for R283A. The ratio $k_{pol,c}/k_{pol,i}$ varies from 430-fold for the selection of C·G against C·T to 45-fold for A·T against A·G, while the ratio $K_{d,i}^{app}/K_{d,c}^{app}$ varies from 20-fold for A·T against A·A to 3-fold for G·C against G·A. The k_{pol} of correct dNTP incorporations catalyzed by R283A decreases from that of WT by a factor up to 520 (for T·A) while the rates of incorrect dNTP incorporations decrease to a lesser degree, which contributes to the lower fidelity of R283A. The fidelity of R283A is reduced from that of WT in all 12 mispairs. The extent of the decrease varies from 200-fold in A·G to 2.5-fold in T·C.

The finding that the R283A mutation affects the fidelity of pol β is significant since the side chain of R283 interacts directly with the template base rather than the incoming dNTP (Pelletier *et al.*, 1994). A possible explanation is described here. As shown in Figure 1, Arg-283 forms a hydrogen bond with the template base opposite the incoming dNTP base. This hydrogen bonding (along with base stacking) could help to position the template base for base pairing with the correct dNTP. Removal of this hydrogen bond should lead to higher entropy to be overcome in the base pairing process, which should in turn lead to a decrease in k_{pol}/K_d^{app} for R283A relative to WT. This effect should be smaller for the misincorporation of incorrect dNTPs since incorrect base pairings are not positioned exactly even for WT. The smaller decreases in k_{pol}/K_d^{app} (for R283A relative to WT) for incorrect dNTPs lead to the reduced fidelity observed for R283A relative to WT.

Mismatch Specificities of WT Pol β and R283A Are Different. For the WT pol β, purine·pyrimidine or pyrimidine·purine mismatches are formed more easily than purine·purine or pyrimidine·pyrimidine mismatches. The fidelity of the former mispairs varies from 3100 to 4500 while that of the latter type of mutation varies from 1600 to 51 000. The T·C mispair was the only one with lower fidelity than purine·pyrimidine or pyrimidine·purine. Therefore, transition mutations are favored over transversion mutations. This result is consistent with what was observed with the forward mutation assay (Kunkel & Alexander, 1986). It has been shown that KF(exo⁻) also displays the same trend (Carroll *et al.*, 1991).

Significant changes in the mismatch specificity occurred upon the conversion of Arg-283 to Ala. Purine·purine mismatches are formed at least as easily as the other types of mispairs. Such a change in the mismatch specificity of R283A relative to that of WT leads to large reductions in the fidelity of purine·purine-type mismatches for R283A, as shown in Figure 6. The reductions in fidelity for other types of mispairing are substantially smaller. A possible explanation for this observation is that the increased overall size of the purine·purine mispair can be more easily accommodated in the binding pocket, which has been expanded by removing the long guanidinium carbon chain.

Asymmetry of Fidelity. Our results have clearly established that the kinetic parameters of the Y·X and X·Y pairs are different. It should be noted that the Y·X and X·Y pairs have different modes of interaction in the binding pocket of polymerases. This is true whether the Y·X and X·Y are correct or incorrect pairs. In the case of mismatches, the fidelity is also different between the Y·X and X·Y pairs.

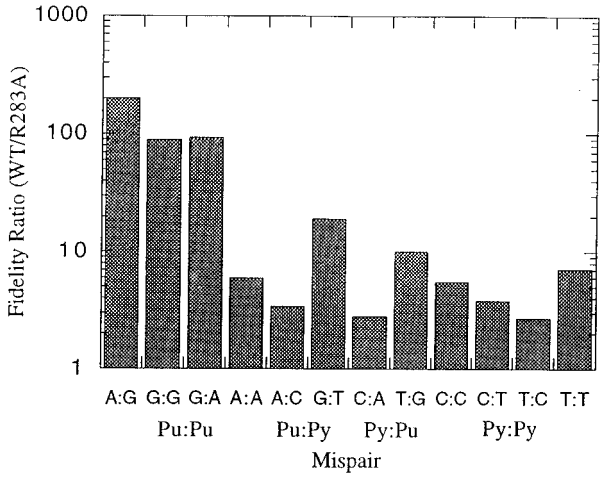

FIGURE 6: Summary of the reduction in fidelity upon replacement of Arg-283 with Ala. The ratio of the fidelity of wild type and R283A for each mispair is shown. Pu and Py represent purine and pyrimidine, respectively.

This is because the active site is clearly asymmetric and the residues interacting with the template base and the incoming dNTP base are different.

Comparison with the Results of Forward Mutation Assays for WT Pol β. The *LacZ* forward mutation assay using M13mp2 as a single-stranded template has been used to study the base substitution fidelity of pol β for 12 possible mispairs (Kunkel & Alexander, 1986). It was found that the enzyme has an average error rate of 1/1500 and the error frequency varies from 1/900 for C·A to 1/22 000 for A·A. The major advantage of the forward mutation assay is that the information about base substitution fidelity over a wide range of DNA base sequences can be obtained and deviations due to mutational hot spots (Kunkel & Alexander, 1986; Bebenek *et al.*, 1990) can be minimized. On the other hand, the fidelity obtained from the forward mutation analysis has a higher error range than the fidelity from the pre-steady-state kinetic analysis. For example, the fidelity in the misincorporation of dTTP opposite template dCMP was estimated to be greater than 9700 from the forward mutation assay, because there was no mutation observed in the 9700 possible sites sequenced. The fidelity for this mispair under the pre-steady-state kinetic assay condition was estimated to be 14 000 at one site of a defined sequence. This value is more accurate, because there is always an accumulation of mismatched product in the pre-steady-state assay. Most importantly, the forward mutation assay cannot provide quantitative rate constants and binding constants for both correct and incorrect nucleotides. These quantitative kinetic constants are required for understanding of the mechanism of fidelity and the structure−fidelity relationship.

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