Variants of DNA Polymerase β Extend Mispaired DNA Due to Increased Affinity for Nucleotide Substrate†

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ABSTRACT: DNA polymerase β offers an attractive system to study the biochemical mechanism of polymerase-dependent mutagenesis. Variants of DNA polymerase β, Y265F and Y265W, were analyzed for misincorporation efficiency and mispair extension ability, relative to wild-type DNA polymerase β. Our data show that the fidelity of the mutant polymerases is similar to wild-type enzyme on a one-nucleotide gapped DNA substrate. In contrast, with a six-nucleotide gapped DNA, the mutant proteins are slightly more accurate than the wild-type enzyme. The mutagenic potential of Y265F and Y265W is more pronounced when encountering a mispaired DNA substrate. Here, both variants can extend a G:G mispair quite efficiently, and Y265F can also extend a T:G mispair. The kinetic basis of the increased mispair extension efficiency is due to an improved ability to bind to the incoming nucleotide. Y265W extends the G:G mispair even with an incorrect nucleotide substrate. Overall, our results demonstrate that the Y265 hinge residue is important for stabilizing the architecture of the nucleotide binding pocket of DNA polymerase β, and that alterations of this residue can have significant impacts upon the fidelity of DNA synthesis.

Mutations resulting from DNA polymerases have been suggested to be a cause of tumor development (*1*, *2*). Mutations can result from DNA polymerase-catalyzed incorporation of the incorrect deoxynucleoside triphosphate (dNTP)[1] substrate. The first step in mutation fixation is extension of a mispaired primer-terminus. In addition, the mispaired nucleotide must escape removal by a proofreading exonuclease and the mismatch repair system to become fixed as a mutation in the genome. Therefore, it is important to understand how polymerases discriminate between the correct dNTP substrate and incorrect ones and how these enzymes prevent extension of a mispaired primer-terminus. Both factors are important contributors to polymerase fidelity.

DNA polymerase β (pol β) offers an attractive system to study the biochemical mechanisms of mutagenesis, because of its small size of 39 kDa and no proofreading activity. This polymerase contains 5′deoxyribose phosphate lyase and polymerase activities, which allow this enzyme to function in short-patch base excision repair (*3*, *4*), by enzymatically removing 5′deoxyribose phosphate and adding one nucleotide to the 3′end of the gap in the DNA (*5*−*7*). Besides short-patch base excision repair, pol β is also a contributor to long-patch base excision repair (*8*−*10*).

To identify amino acid residues of pol β that are critical for accurate DNA synthesis, we developed a genetic screen (*11*). This genetic screen has identified several mutator mutants of pol β, some of which have been characterized in vivo and in vitro (*12*−*17*). One of these mutants, Y265H, displays a 120-fold loss in fidelity relative to wild-type pol β (WT) (*17*). The structural basis of the fidelity loss is most likely due to an altered geometric alignment of active site residues, the DNA template, the primer terminus, the incoming dNTP substrate, and the catalytic Mg ions. This leads to a rate of polymerization that is only 8-fold faster for the correct nucleotide than the incorrect substrate. Two other variants of residue 265, Y265F and Y265W, were analyzed by a forward mutation assay (*18*). This analysis indicates that Y265W has a tendency to incorporate multiple mutations at 56 times greater frequency than WT. To examine the molecular basis for the phenotype of the Y265 variants, we used pre-steady-state kinetic analysis to characterize the ability of these variants to misincorporate nucleotides and evaluated their ability to extend a mispaired primer-terminus in the presence of either the correct or incorrect dNTP substrate. Our results indicate a similar misincorporation fidelity to WT for Y265F and Y265W on a one-nucleotide gapped DNA substrate, whereas on a six-nucleotide gapped DNA substrate, the two mutant polymerases are a little more accurate than WT. In the presence of a mispaired DNA substrate, Y265 variant enzymes extend the mispair with greater polymerization efficiency than WT with the next correct dNTP substrate. Y265W also can extend the mispair with an incorrect nucleotide substrate. The apparent increase in mispair extension efficiency appears to be due to an enhanced affinity for the incoming nucleotide substrate for the Y265 mutant polymerases. Thus, our results suggest the importance of Tyr-265 of pol β in maintaining

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[1] Abbreviations: deoxynucleoside triphosphate, dNTP; pol β, DNA polymerase β; Y265F, Y265F mutant of DNA polymerase β; Y265W, Y265W mutant of DNA polymerase β; Y265H, Y265H mutant of DNA polymerase β; WT, wild-type DNA polymerase β.

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45AG-22C-22     5'-GCCTCGCAGCCGTCCAACCAAC CAACCTCGATCCAATGCCGTCC
                3'-CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG

45AG-22C-17     5'-GCCTCGCAGCCGTCCAACCAAC      TCGATCCAATGCCGTCC
                3'-CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG

45AG-22G-17     5'-GCCTCGCAGCCGTCCAACCAAG      TCGATCCAATGCCGTCC
                3'-CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG

45AG-22T-17     5'-GCCTCGCAGCCGTCCAACCAAT      TCGATCCAATGCCGTCC
                3'-CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG
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FIGURE 1: Gapped DNA substrates. The DNA substrates used for incorporation of T opposite template A as well as misincorporation of G opposite A. All of the oligonucleotides were phosphorylated at the 5′ends. Substrates were prepared as described in Experimental Procedures. 45AG-22C-22 is the one-nucleotide gapped DNA, 45AG-22C-17 is the six-nucleotide gapped DNA, 45AG-22G-17 is the six-nucleotide gapped DNA, which contains a G:G mispair at its primer terminus, and 45AG-22T-17 is the six-nucleotide gapped DNA that contains a T:G mispair at its primer terminus.

proper geometric alignment of the active site residues, the DNA, Mg ions, 3′OH of the primer, and the incoming dNTP substrate for both misincorporation fidelity and the prevention of mispair extension.

EXPERIMENTAL PROCEDURES

Materials. Deoxynucleoside triphosphates were purchased from New England Biolabs (Beverly, MA). Adenine triphosphate was purchased from Sigma. [γ^{32}P]ATP was purchased from Amersham. The recombinant rat wild-type pol β was purified as described (*17*). Y265F and Y265W were prepared as described (*18*). Synthetic oligonucleotides were purchased from HHMI Biopolymer and W. M. Keck Biotechnology Resource Laboratory at Yale University. The concentration of protein in each preparation was based on an $\epsilon 280 = 21\,200\ M^{-1}\ cm^{-1}$ and a molecular mass of 40 kDa for His-tagged pol β.

Preparation of Gapped DNA Substrates. Denaturing polyacrylamide gel electrophoresis was used to purify the oligonucleotides and concentrations were determined by UV absorbance at 260 nm. The extinction coefficients used to estimate the concentrations of oligonucleotides were as follows: 45 mer = 431 000 M^{-1} cm^{-1}; 22C-mer = 197 700 M^{-1} cm^{-1}; 22-mer = 198 500 M^{-1} cm^{-1}; 17-mer = 153 200 M^{-1} cm^{-1} 22G-mer = 201 500 M^{-1} cm^{-1}; 22T-mer = 199 300M^{-1} cm^{-1}. We used T4 polynucleotide kinase to catalyze the addition of [γ^{32}P]ATP to each of the oligonucleotides according to manufacturer's instructions. The primer strand was labeled with [γ^{32}P]ATP, while the other oligonucleotides were labeled with nonradioactive ATP. Unincorporated nucleotides were removed using a P-30 or P-6 microspin column (Bio-Rad).

Gapped DNA substrates were formed by annealing an equimolar ratio of radioactive primer, downstream oligomer, and template in 50 mM Tris-Cl, pH 8.0 containing 0.25 M NaCl. The mixture was incubated sequentially at 95 °C (5 min), slow cooled to 50 °C (30 min), 50 °C (20 min), and immediately transferred to ice. To examine the influence of gap size on nucleotide discrimination for WT, Y265F, and Y265W, two substrates were constructed with the same [5′-^{32}P] 22-mer primer hybridized to a DNA template of 45 nucleotides (Figure 1). This places the 3′OH primer terminus such that incorporation of the next dNTP occurs opposite template A. Preparation of the one-nucleotide gapped substrate, 45AG-22C-22, was performed with the radiolabeled primer, template, and a phosphorylated oligomer of 22 nucleotides, whereas the six-nucleotide gapped substrate, 45AG-22C-17, contained a 5′phosphorylated strand of 17 nucleotides downstream from the primer. Proper primer annealing was confirmed by nondenaturing polyacrylamide gel electrophoresis on an 18% gel. Gapped DNA was utilized, since this type of structure is probably most physiologically relevant (*19*, *20*). To study the ability of the polymerase to extend mispairs, two other six-nucleotide gapped substrates, 45AG-22G-17 and 45AG-22T-17, were prepared. These substrates are the same as the 45AG-22C-17, except with a G or T at the 3′end of the radiolabeled 22-mer primer described above (Figure 1).

Rapid Quench-Flow Experiments. A KinTek Instruments model RQF-3 rapid-quench-flow apparatus (*21*) was used for rapid chemical quench flow experiments. Reactions were conducted in buffer (50 mM Tris-Cl buffer (pH 8.0) containing 10 mM $MgCl_2$, 2 mM DTT, 20 mM NaCl, and 10% glycerol) at 37 °C. Protein concentrations were based on active-site titration as described (*22*−*24*), unless noted. The WT preparation had 45% active sites, Y265F had 38% active sites, and Y265W had 40% active sites. A typical experiment was carried out by preincubating pol β and gapped DNA in buffer in the absence of Mg^{2+} ion. A 15 μL aliquot of this solution was rapidly mixed with an equal volume of Mg-nucleotide solution. The reactions were quenched with 0.1 M EDTA at a specified time interval, ranging from 0.005 to 60 s. All concentrations reported refer to the concentrations after mixing.

Measurement of Equilibrium Dissociation Constant of Nucleotide (K_d) and the Maximum Rate of Polymerization (k_{pol}). To determine the K_d and k_{pol}, incorporation of dTTP (correct) and dGTP (incorrect) opposite template A was examined as a function of time as previously described (*17*, *22*, *23*). In these experiments, a solution containing a preincubated complex of pol β enzyme (500 nM) and radiolabeled gapped DNA (50 nM) was mixed with a solution of $MgCl_2$ (10 mM) and varying concentrations of a single dNTP. These conditions allow the rate of a single catalytic turnover of the enzyme to be measured. The reaction was stopped with EDTA at various times. Experiments for dTTP incorporation were performed on the KinTek apparatus, whereas misincorporation of dGTP was performed manually under identical reaction conditions. Manual kinetic experiments were performed as described (*17*). All nucleotide incorporation reactions were performed at 37 °C.

Mispair Extension Assay. Extension of the G:G and T:G mispaired primer-termini was performed under single turn-

over conditions using a six-nucleotide gapped DNA substrate in the presence of either correct or incorrect dNTP substrate. The single turnover conditions were determined empirically, by titration of increasing concentrations of mispaired primer-template with enzyme. A ratio of 15:1 (enzyme/DNA) was chosen for each reaction.

Product Analysis. The DNA products were resolved by sequencing gel electrophoresis under denaturing conditions (20% acrylamide, 8 M urea, and 1× TBE running buffer) and quantified using a PhosphorImager (Molecular Dynamics Storm 840), as described (*17*).

Data Analysis. Data were analyzed by nonlinear regression using the program KaleidaGraph (Synergy Software). Data from burst experiments were fit to the equation: [product] $= A[1 - \exp(-k_{obs}t)] + k_{ss}t$, where A is the amplitude of the burst, k_{obs} is observed first-order rate constant for dNTP incorporation, and k_{ss} is the observed linear rate constant. Single turnover kinetic data were fitted to a single-exponential equation: [product] $= A[1 - \exp(-k_{obs}t)]$, where A represents the amplitude, and k_{obs} is the observed rate constant. The observed rate constants were then plotted against [dNTP], and the data were fitted to the hyperbolic equation: $k_{obs} = k_{pol}$[dNTP]/(K_d + [dNTP]), where k_{pol} is the maximum rate of polymerization and K_d is the equilibrium dissociation constant for dNTP. Fidelity (F) values were calculated using the relationship: $F = [(k_{pol}/K_d)_c + (k_{pol}/K_d)_i]/(k_{pol}/K_d)_i]$, where c and i represent the correct and incorrect dNTP substrate, respectively.

Interaction of pol β with DNA. The equilibiruim dissociation constant, K_d, for the interaction of pol β with DNA was obtained using a gel mobility shift assay for six-nucleotide gapped DNA binding in the presence and absence of the mispairs, because these enzymes do not exhibit burst kinetics in the presence of a mispair. Pol β protein (0.1−1000 nM) was incubated with 0.1 nM radiolabeled gapped DNA substrate in buffer containing 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and 0.1% Nonidet P-40 at room temperature (23 °C) for 15 min. Samples were centrifuged for 30 s and loaded onto a 6% native polyacrylamide gel with the current running at 300 V at 4 °C. After the sample was loaded, the voltage was reduced to 150 V. Bound protein was quantified using Imagequant software, after scanning the gel using a Molecular Dynamics Phosphorimager. Protein bound to DNA resulted in a shift of the DNA on the gel such that it migrated less rapidly than DNA without protein bound to it. To quantify bound protein, the band intensity of all shifted species from band A in Figure 6A to the top of the gel, band D, was measured for each lane by obtaining the intensity of a rectangular box drawn to accommodate the width of the lane and with dimensions extending from band A to band D. The same box was used to measure the intensity of shifted species in each lane of the gel. This intensity was added to the intensity of the unshifted species at the bottom of the gel in Figure 6A to obtain the total. Fraction bound is the ratio of the intensity of all shifted species divided by the total. The dissociation constant for DNA (K_D) was estimated from fitting the bound protein (Y) versus protein concentration (x) with the equation: $Y = [(mx)/(x + K_D)] + b$, where m is a scaling factor and b is the apparent minimum Y value.

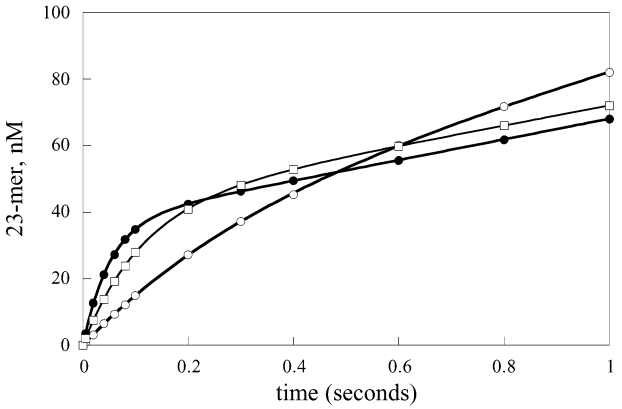

FIGURE 2: Incorporation of dTTP opposite template A. Insertion of dTTP into a six-nucleotide, gapped DNA substrate was measured using the chemical quench-flow apparatus. A preincubated solution of 100 nM enzyme (based on absorbance at 280 nm) and 300 nm gapped DNA was mixed with a solution of dTTP (a concentration equaling 5 times the K_d for dTTP of each polymerase) and 10 mM $MgCl_2$. Reactions were quenched with EDTA, and the product was resolved by denaturing gel electrophoresis. Data were fit to the burst equation. For WT (○), the $k_{obs} = 3.1 \pm 0.2$ s^{-1} and the steady-state rate constant is 1.1 s^{-1}. For Y265F (●), the $k_{obs} = 19.4 \pm 0.8$ s^{-1} and the steady-state rate constant is 1.2 s^{-1}. For Y265W (□), the $k_{obs} = 9.0 \pm 0.4$ s^{-1} and the steady-state rate constant is 1.4 s^{-1}.

RESULTS

Pol β Proteins Display Biphasic Kinetics on a Six-Nucleotide Gapped DNA. To determine the mechanism of polymerization of single-nucleotide incorporation by WT, Y265F, and Y265W on a six-nucleotide gapped DNA substrate, we first examined the incorporation of correct nucleotide, dTTP, opposite a template A residue in a burst experiment. The amount of 23-mer product generated was plotted as a function of time for all three pol β enzymes (Figure 2). The kinetics of nucleotide incorporation were biphasic, where a rapid burst phase was followed by a slow linear phase (rates are shown in the legend of Figure 2). For WT, unlike the Y265 mutant polymerases, the two phases are almost indistinguishable, because the rate of the burst phase is similar to the rate of the linear phase. The biphasic kinetic profile is more defined for WT on a single-nucleotide gapped DNA substrate with the same sequence (*17*). Thus, for all three polymerases, the rate-limiting step of single-nucleotide incorporation is after the chemical step, which is most likely dissociation of the DNA from pol β. However, for six-nucleotide gapped DNA, there is not a well-defined burst for WT, suggesting that chemistry and product release are partially rate-limiting.

Y265 Variants Misincorporate on a One-Nucleotide Gapped DNA. Under single turnover conditions, the kinetics for single nucleotide incorporation catalyzed by several DNA polymerases (*23*−*26*), including pol β (*27*, *28*), follows the pathway shown below, where k_{pol} is the maximum rate of polymerization and K_d is the ground-state nucleotide equilibrium dissociation constant.

$$\text{E-DNA}_n + \text{dNTP} \overset{K_d}{\rightleftharpoons} \text{E-DNA}_n\text{-dNTP} \overset{K_{pol}}{\rightleftharpoons} \text{E-DNA}_{n+1}\text{-PP}_i$$

Single turnover conditions minimize DNA association and

dissociation by the enzyme, because nearly all of the DNA substrate is occupied by polymerase.

To study misincorporation on a one-nucleotide gapped DNA, we determined the ability of Y265F and Y265W to incorporate an incorrect dNTP substrate, dGTP, versus a correct nucleotide substrate, dTTP, opposite template A. Even though the phenotype of these mutants was obtained on DNA that consists of a very large gap, we decided to use single nucleotide gapped DNA because it is a more physiologically relevant DNA substrate for pol β. Selection of G opposite A was based on a forward mutation assay, which showed that this transversion mutation was observed for Y265W (*18*). We used a 10:1 molar ratio of active enzyme to DNA to monitor the kinetics of a single nucleotide incorporation opposite template A.

The K_d and k_{pol} values were determined by measuring the rate of product formation at varying concentrations of dNTP. Figure 3A illustrates an example of dTTP incorporation opposite A for Y265F at several concentrations of nucleotide at 37 °C. By fitting each set of data to the single-exponential rate equation, the k_{obs} was determined for each dTTP substrate concentration. These values were plotted against the dTTP concentrations to yield the K_d and k_{pol} parameters. An example for Y265F is shown (○) in Figure 3B. The values of k_{pol} and K_d for Y265F were 18.2 s^{-1} and 63 μM, respectively. Values for all the kinetic parameters for the three pol β polymerases on a one-nucleotide gapped DNA are listed in Table 1.

The k_{pol} and K_d rate constants were used to calculate the fidelities for the three proteins. A 2-fold loss in fidelity was observed for Y265F relative to WT. This loss is primarily due to a decreased ability to discriminate between correct versus incorrect dNTP at the level of k_{pol}. The maximum rate of polymerization of the correct dNTP is 4526 times faster than incorrect one, whereas Y265F displays an 1145-fold difference. Thus, Phe substitution for Tyr-265 causes about a 4-fold (4526/1145) loss in discrimination at the level of k_{pol}. The discrimination factor at the level of ground-state binding was similar for Y265F (12; 774/63) and WT (7; 439/65). Y265W shows about a 10% loss in fidelity in comparison to WT. Just like Y265F, Y265W has a reduced ability to discriminate dTTP from dGTP at the level of k_{pol}. Here, the amino acid substitution results in about a 2.4-fold (4526/1921) difference at the k_{pol} level. Nucleotide discrimination is increased at the dNTP binding step by about 2-fold. In addition, Y265W has a higher polymerization efficiency, k_{pol}/K_d, than WT by an order of magnitude for both G and T incorporation opposite template A. Overall, both Y265 variants have similar misincorporation fidelities on a one-nucleotide gapped DNA substrate.

Y265 Mutant Polymerases Have Higher Base Substitution Fidelity than WT on a Six-Nucleotide Gapped DNA. To examine the impact of gap size on nucleotide discrimination, a six-nucleotide gapped DNA substrate was prepared. We analyzed by using single turnover kinetics the ability of WT and Y265 variants to discriminate between dTTP and dGTP. The observed single turnover rates by WT, Y265F, and Y265W were plotted versus the dTTP concentrations. Figure 3B shows an example of the hyperbolic plot for Y265F (●). The values of k_{pol} and K_d for Y265F were 31.7 s^{-1} and 204 μM, respectively. All the kinetic values are listed in Table 2.

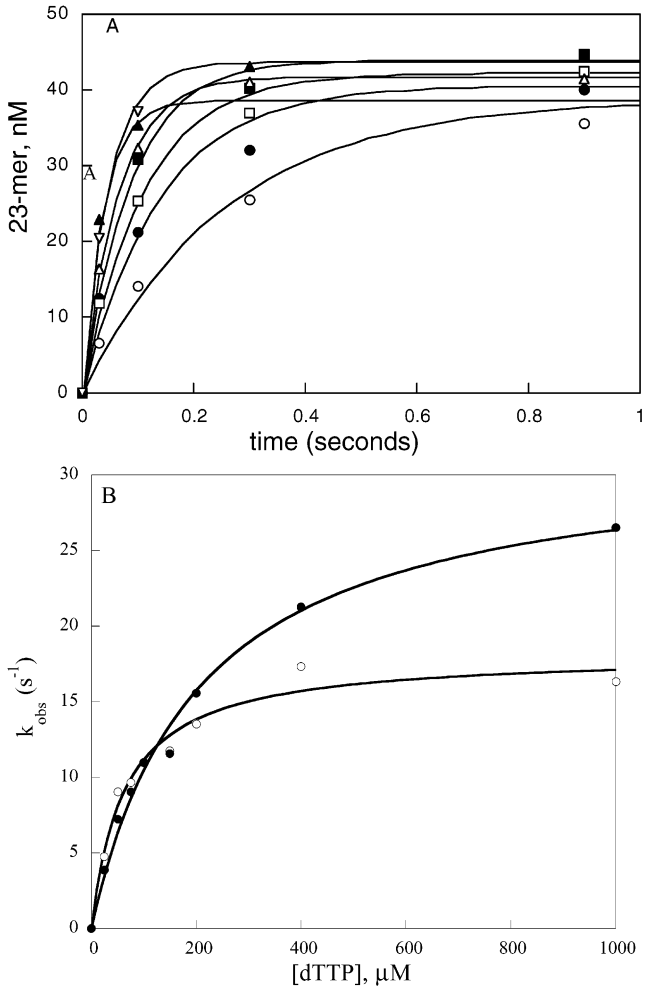

FIGURE 3: Single turnover kinetics of dTTP incorporation opposite template A. (A) Incorporation of dTTP opposite A for Y265F. A preincubated solution containing enzyme (500 nM) and one-nucleotide gapped DNA (50 nM) was mixed with $MgCl_2$ (10 mM) and 25 (○), 50 (●), 75 (□), 150 (■), or 200 (Δ), 400 (▲), or 1000 (∇), μM dTTP. The reactions were terminated and monitored as described in Figure 2. Data were fit to the single-exponential equation to obtain k_{obs}. (B) Hyperbolic plot of k_{obs} against dTTP concentration for Y265F. The data for one-nucleotide gapped DNA (○) and six-nucleotide, gapped DNA substrate (●) were fit to the hyperbolic equation as described in Experimental Procedures. The solid line represents the best fit of the data to the hyperbolic equation. Values of K_d and k_{pol} are listed in Table 1 for one-nucleotide gapped DNA and Table 2 for six-nucleotide gapped DNA.

A striking result was observed for Y265W on the six-nucleotide gapped DNA. With this DNA structure, the fidelity of Y265W (52 300) was about 4-fold higher than WT (13 700). This is primarily due to about a 3-fold (22/7) enhancement of discrimination between correct and incorrect dNTP substrates at the level of K_d. Much of this improved dis-crimination results from the enhanced affinity of Y265W for dTTP opposite template A on six-nucleotide gapped DNA. This same property is enhanced for Y265W on single nucleotide gapped DNA, suggesting that Y265W is a more catalytically efficient enzyme that WT. The fidelity of Y265F is about 2-fold (26 800/13 700) greater than WT with the six-nucleotide gapped DNA. This suggests nucleotide discrimination is similar for WT and Y265F with the six-nucleotide gapped DNA. The Y265W polymerase has an improved ability when compared to WT to discriminate between the

Table 1: Kinetic Values on a One-Nucleotide Gapped DNA

	k_{pol} (s^{-1})	K_d (μM)	$k_{pol(c)}/k_{pol(i)}$[c]	$K_{d(i)}/K_{d(c)}$[d]	k_{pol}/K_d (M^{-1} s^{-1})	F ($\times 10^3$)[e]
			WT			
A:T[a]	8.6 ± 0.4	65 ± 9			1.32×10^5	
A:G[b]	0.0019 ± 0.0002	439 ± 87	4526	7	4.33	30.6
			Y265F			
A:T	18.2 ± 0.9	63 ± 11			2.89×10^5	
A:G	0.0159 ± 0.0012	774 ± 135	1145	12	20.5	14.1
			Y265W			
A:T	19.4 ± 0.3	14 ± 1			1.39×10^6	
A:G	0.0101 ± 0.0011	209 ± 53	1921	15	48.3	28.7

[a] Kinetic values for incorporation of dTTP opposite template A. [b] Kinetic values for misincorporation of dGTP opposite template A. [c] The k_{pol} for correct (c) divided by incorrect (i). [d] The K_d for incorrect (i) dNTP divided by correct (c). [e] Fidelity (F) was calculated as described in Experimental Procedures.

Table 2: Kinetic Values on a Six-Nucleotide Gapped DNA

	k_{pol} (s^{-1})	K_d[c] (μM)	$k_{pol(c)}/k_{pol(i)}$[d]	$K_{d(i)}/K_{d(c)}$[e]	k_{pol}/K_d (M^{-1} s^{-1})	F ($\times 10^3$)[f]
			WT			
A:T[a]	3.4 ± 0.3	112 ± 21			3.04×10^4	
A:G[b]	0.0017 ± 0.0001	768 ± 117	2000	7	2.21	13.7
			Y265F			
A:T	31.7 ± 1.1	204 ± 21			1.55×10^5	
A:G	0.0034 ± 0.0003	593 ± 103	9323	3	5.7	26.8
			Y265W			
A:T	14.4 ± 1.1	41 ± 8			3.51×10^5	
A:G	0.0061 ± 0.0007	903 ± 204	2360	22	6.75	52.3

[a] Kinetic values for incorporation of dTTP opposite template A. [b] Kinetic values for misincorporation of dGTP opposite template A. [c] Units are μM. [d] The k_{pol} for correct (c) divided by incorrect (i). [e] The K_d for incorrect (i) dNTP divided by correct (c). [f] Fidelity (F) was calculated as described in Experimental Procedures.

dNTP substrates with the six-nucleotide gapped DNA substrate.

Y265 Variants Extend a G:G Mispair More Efficiently than WT. The ability to extend a mispair was also compared between the mutant enzymes and wild-type pol β under single-turnover conditions. An initial survey of all the mispairs showed that the Y265 variants extended a G:G and T:G mispair much better than WT. However, the mutants did not catalyze misinsertion of dGTP opposite G or dTTP opposite G with efficiencies significantly different than WT (data not shown). To probe the mechanistic basis of mispair extension, a six-nucleotide gapped DNA substrate that contained a G:G mispair at the primer terminus was used. Time courses were conducted at different concentrations of the next correct nucleotide substrate, dTTP, for each of the three polymerases under single turnover conditions. An example is shown for Y265W (Figure 4A). The observed rate constant was then plotted against dTTP concentration and the data were fit to the hyperbolic equation. Figure 4B shows an example of the hyperbolic plot obtained for G:G mispair extension with dTTP by Y265W. The values of k_{pol} and K_d were 0.021 s^{-1} and 5.5 μM, respectively, for Y265W. Values for WT, Y265F, and Y265W are listed in Table 3 for G:G mispair extension with dTTP.

On the basis of the single-turnover kinetic data, extension of the G:G mispair with correct nucleotide substrate is most efficient with Y265W, followed by Y265F. The polymerization efficiency (k_{pol}/K_d) for Y265W is about 50 (3818/77)

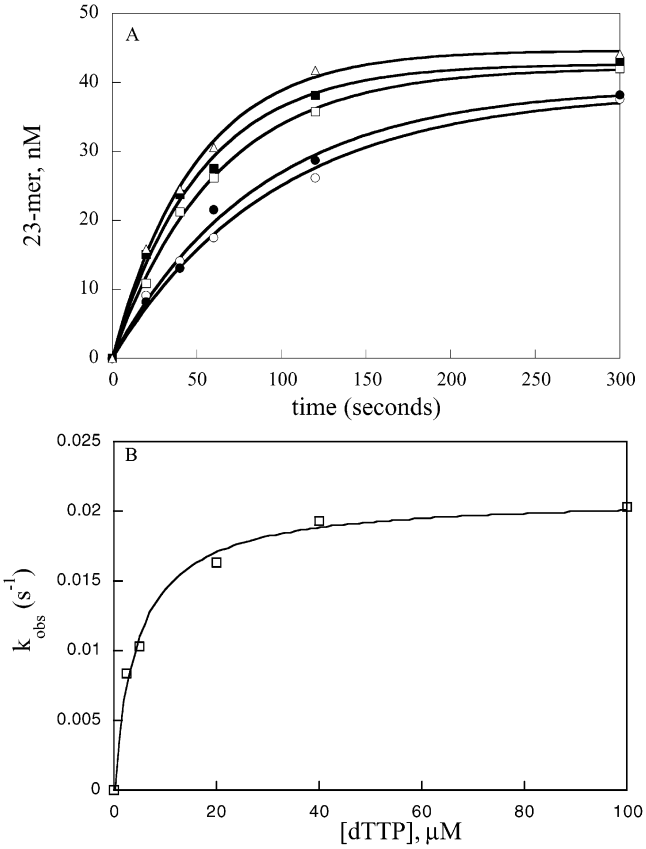

FIGURE 4: Extension of a mispaired, six-nucleotide gapped DNA with the next correct nucleotide. (A) Time courses of dTTP insertion by Y265W (500 nM) opposite template A onto a six-nucleotide, gapped DNA (50 nM) that contains a G:G mispair at the primer terminus. The final concentrations of dTTP were 5 (○), 10 (●), 20 (□), 40 (■), and 100 (Δ) μM. The data were fit to a single-exponential rate equation to obtain k_{obs}. (B) Hyperbolic plot of Y265W-dependent extension of mispaired DNA. The observed rate constants were plotted against the corresponding dTTP concentration and fit to the hyperbolic equation. Values for k_{pol} and K_d for each of the pol β enzymes are shown in Table 3.

Table 3: Y265 Variants Extend a G:G and T:G Mispaired Primer-Terminus

base pair	enz	k_{pol} (s^{-1})	K_d (μM)	$k_{pol(c)}/k_{pol(I)}$[c]	$K_{d(I)}/K_{d(c)}$[d]	k_{pol}/K_d (M^{-1} s^{-1})
C:G[a]	WT	3.4 ± 0.3	112 ± 21			3.04×10^4
	Y265F	31.7 ± 1.1	204 ± 21			1.55×10^5
	Y265W	14.4 ± 1.1	41 ± 8			3.50×10^5
G:G[b]	WT	0.022 ± 0.001	284 ± 24	155	2.5	77
	Y265F	0.038 ± 0.001	56 ± 7.0	83	0.27	679
	Y265W	0.021 ± 0.001	5.5 ± 1.2	686	0.13	3818
T:G[e]	WT	0.028 ± 0.001	33 ± 1.1	121	0.29	851
	Y265F	0.582 ± 0.035	44 ± 7.7	55	0.21	1.3×10^4
	Y265W	0.149 ± 0.025	251 ± 65	97	6.1	593

[a] Kinetic values for extension of the C:G correct pair by incorporation of dTTP opposite template A. [b] Kinetic values for extension of the G:G mispair by incorporation of dTTP opposite template A. [c] The k_{pol} for correct (c) divided by incorrect (i). [d] The K_d for incorrect (i) dNTP divided by correct (c). [e] Kinetic values for extension of the T:G mispair by incorporation of dTTP opposite template A.

times greater than WT and about 6 (3818/679) times greater than Y265F. The basis of the higher extension efficiency is due to about a 52-fold (284/5.5) increase in ground-state binding of the correct nucleotide substrate for Y265W, when compared to WT. In essence, there is nearly a total lack of discrimination during the binding step for Y265W. Following

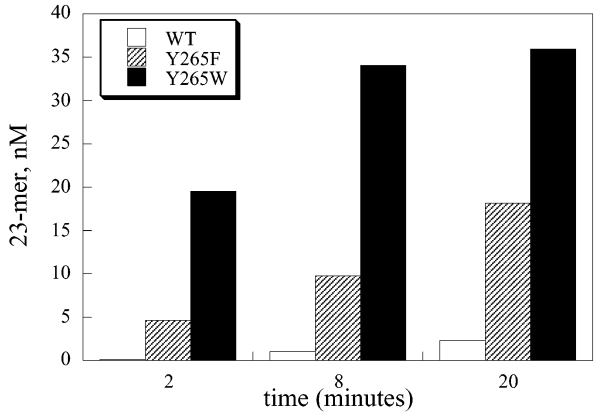

FIGURE 5: Extension of a mispaired, gapped DNA with the next incorrect nucleotide. Incorporation of dGTP opposite template A onto a six-nucleotide, gapped DNA (50 nM) that contains a G:G mispair at the primer terminus. The DNA substrate was incubated with 500 nM WT, 500 nM Y265F, or 500 nM Y265W for 2, 8, or 20 min in the presence of dGTP (2 mM). Products were resolved by denaturing polyacrylamide gel electrophoresis and visualized using a phosphorimager. Extension of [5′-^{32}P]22-mer primer to 23-mer corresponds to the insertion of G opposite template A. Amount of product, 23-mer, as quantified by phosphorimager software for WT (clear), Y265F (hatched), and Y265W (filled) for extension of G:G with incorrect dNTP substrate at indicated reaction times.

the same pattern as Y265W, Y265F shows about a 9-fold (679/77) increase in efficiency compared to WT in extending the G:G mispair with dTTP. This increase is due to about a 5-fold (284/56) increase in nucleotide affinity for Y265F, when compared to WT.

To determine if the Y265F and Y265W variants were able to extend another mispair, we performed similar experiments with a T:G mispair. Specifically, we asked whether Y265F and Y265W could extend a T:G mispair using the substrate 45 AG-22T-17. The results in Table 3 show that Y265F is able to extend the T:G mispair with catalytic efficiency that is nearly 10% of the extension of a normal C:G base pair. Extension of the T:G mispair by Y265F appears to result from a lack of discrimination at the level of k_{pol} when compared to WT. WT extends the T:G mispair at an efficiency that is 10-fold greater than extension of the G:G mispair. Y265W extends the T:G mispair at an efficiency that is nearly equal to that of WT, but is about 6-fold less than extension of the G:G mispair.

The ability to extend a G:G mispair in the presence of dGTP, the incorrect nucleotide substrate, was compared between the three polymerases. Figure 5 shows the amount of product formed for each polymerase at 2, 8, and 20 min reaction times. With WT, there is very little product generated, even at 20 min. However, a striking result shows that Y265W, and, to a lesser extent, Y265F, incorporate dGTP to form the 23-mer product. Thus, the Y265 mutant enzymes can extend the G:G mispair with an incorrect nucleotide substrate. Analysis of dGTP titration could only be performed for Y265W, since the WT could not extend the mispair and Y265F could extend the mispair to about 36% in 20 min at 2 mM dGTP. A complete dGTP titration for Y265W-dependent extension of the G:G mispair with incorrect dNTP substrate gives a k_{pol} of 0.0082 ± 0.0007 s^{-1}, K_d of 278 ± 49 μM, and a k_{pol}/K_d of 30 M^{-1} s^{-1}. The polymerization efficiency, (k_{pol}/K_d), of misincorporation of G opposite template A is about 4-fold (30/6.7) higher for a mispaired substrate than a correctly paired one. The fidelity for discrimination of G versus T opposite template A on the mispaired, six-nucleotide gapped DNA is 130 for Y265W. In comparison to a correctly paired, six-nucleotide gapped DNA, the fidelity is about 400 (52 300/130) times lower for the mispaired, gapped DNA substrate. These data suggest that Y265W loses the ability to discriminate correct from incorrect dNTP substrates in the presence of a G:G mispaired, six-nucleotide gapped DNA.

Y265W, Y265F, and β-WT Have Similar Affinity for Six Nucleotide Gapped DNA in the Presence or Absence of a Mispair. A plausible explanation for the ability of the Y265F and Y265W variants to extend a mispaired primer-terminus is that they have increased affinity over WT for DNA with a mispaired primer terminus. We employed a gel shift assay to estimate the apparent equilibrium dissociation constant, K_D, for WT and the mutants. This assay was used instead of active site titration because the WT and mutants do not exhibit a rapid burst of product formation in the presence of a mispaired primer-terminus. A scan of the gel shift assays for WT, Y265F, and Y265W is shown in Figure 6A, along with a plot of the fraction of enzyme bound DNA verses various concentrations of enzyme in Figure 6B. As can be seen for all enzymes, a shifted band is detected only when enzyme is incubated with DNA. A shifted species, band A, is observed when the concentration of protein in the reaction ranges from 0.244 to 31.25 nM (lanes 2−7) for each of the enzymes. Therefore, only band A is observed when the fraction bound approximates 0.1−0.65 (Figure 6B). Additional smeared bands B and C are observed for Y265W at 31.25 and 62.5 nM protein, respectively, when the fraction bound is ∼0.60 and greater. Smeared bands B and C are also observed for WT but not Y265F. The slowest migrating band D is observed for all three proteins beginning at 250 nM (lane 10), 62.5 nM (lane 8), and 125 nM (lane 9) for WT, Y265W, and Y265F, respectively, and continues to be observed until the concentration of enzyme in the reaction is 1000 nM (lane 12). Therefore, the most slowly migrating DNA−protein species appears to form when the fraction bound is ∼0.60 or greater. We are uncertain of the molecular nature of each of the protein−DNA species, although we suspect that bands B, C, and D consist of a DNA−protein aggregate of pol β, because enzyme is in vast excess of DNA when these bands are observed. Qualitatively, it appears that the gel shift profiles of the variants and WT are similar. This is supported by the plots of the gel shift data in Figure 6B, which are also quite similar.

As shown in Table 4, the K_D values for WT and the Y265 variants for DNA containing a correctly paired primer terminus (C:G) and mispaired primer-termini (T:G, G:G) are within a factor of 2. Because the apparent K_D's of the WT and mutant proteins for DNA are similar, these data suggest that the affinity of these enzymes for DNA does not provide an explanation for the ability of the Y265 variants to extend mispaired primer-termini.

DISCUSSION

Extensive studies on the fidelity properties of DNA polymerases over the past three decades have provided evidence that the DNA polymerase itself participates in dNTP substrate selection (*21*, *29*). DNA polymerases choose the

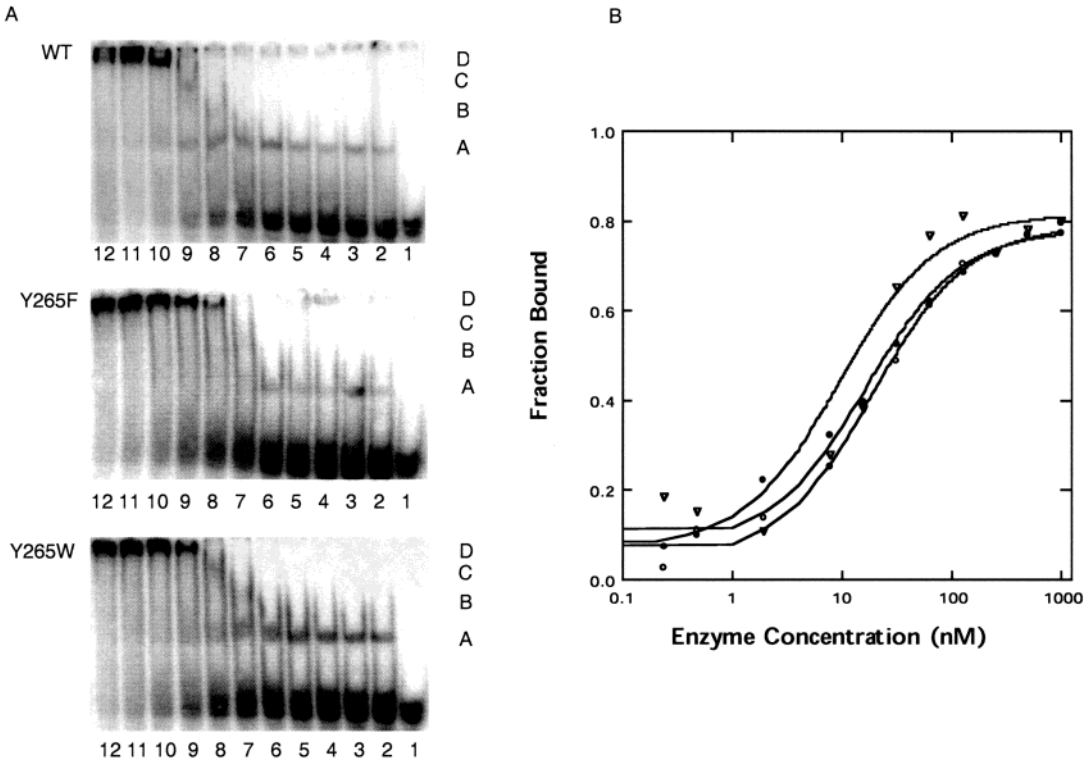

FIGURE 6: Gel shift assays for pol β-WT, Y265W, and Y265F. (A) Scans of the gels. Lanes shown represent reactions containing 0.1 nM DNA and 0, 0.244, 0.488, 1.95, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 nM enzyme (concentration based on A280) in lanes 1−12, respectively. As described in Experimental Procedures, 0.1 nM radiolabeled DNA was incubated with various concentrations of enzyme at room temperature for 15 min. Samples were resolved on a 6% polyacrylamide gel. Bands obtained by incubating DNA with protein are labeled as A, B, C, D, on the right side of each gel scan. (B) Plots of the fraction of DNA bound vs the concentration of enzyme. (∇) WT, (●) Y265W, (○) Y265F. The Sigmaplot graphics program was used to plot the data, and the data were fit as described in Experimental Procedures to obtain the K_D.

Table 4: The Apparent Equilibrium Dissociation Constants for Correctly Paired and Mispaired DNA Are Similar for the WT and the Y265F and Y265W Variants[a]

	K_D (nM)		
pol β protein	C:G[b]	T:G[c]	G:G[d]
WT	18 ± 1.0	9.6 ± 2.1	15 ± 5.0
Y265F	11 ± 0.4	16 ± 2.0	16 ± 2.8
Y265W	16 ± 2.0	19 ± 2.0	8.7 ± 1.6

[a] The data presented are representative from at least three experiments with WT and each of the variants. [b] A primer-terminus with a C:G mispair. [c] A primer-terminus with a T:G mispair. [d] A primer-terminus with a G:G mispair. The DNA substrates are all six-nucleotide gapped DNA.

correct substrate from a group of incorrect ones in a highly precise manner. When a polymerase does occasionally incorporate an incorrect dNTP substrate, the subsequent mispair needs to be extended and to evade removal by exonucleases and DNA repair systems. Therefore, it is important to understand how polymerases choose the correct dNTP substrates from incorrect ones. Equally important is trying to understand the mechanisms that DNA polymerases use to extend a mispaired primer terminus.

Our main objective in this study was to examine the kinetics of the Y265F and Y265W mutants of pol β during misincorporation and mispair extension. We compared the abilities of the mutant polymerases and wild-type pol β to misincorporate nucleotides and to extend a mispaired primer terminus with both correct and incorrect nucleotide. We found that the Y265W and Y265F variants have slightly increased fidelities over WT on six-nucleotide gapped DNA. We also demonstrated that the Y265W and Y265F mutants are able to extend mispaired primer-termini with an increased catalytic efficiency over that of WT. Because both of these enhanced properties of the mutants relate to their interaction with various types of DNA substrates, we conclude that the mutants interact with certain DNA substrates in a manner different than WT. However, our DNA binding studies indicate that there is no quantitative difference in the equilibrium dissociation constants of these enzymes for DNA. This suggests that the altered interaction of Y265W and Y265F with DNA is associated with the positioning of the DNA within the enzyme, rather than with the affinity of the enzyme for the DNA.

Y265W Is more Accurate than WT with Six-Nucleotide Gapped DNA Substrate. The mechanistic basis for the increased accuracy of Y265W on six-nucleotide gapped DNA resides in the kinetic parameter of ground-state binding. This suggests that Y265W is able to discriminate the correct from the incorrect dNTP substrate somewhat better than WT during the initial dNTP-binding step, when the enzyme may be in an open form.

In the structure of the closed form of pol β (1BPY, Protein Data Base), the incoming nucleotide is wedged between amino acid residues of pol β and DNA. These residues include Tyr-271, Phe-272, Asn-279, Asp-276, which are important for discrimination during ground-state binding, and Arg-183 and Ser-180, which interact with the phosphate moieties of the substrate (*15*, *30*−*34*). Part of the binding pocket also consists of DNA template and the 3′-end of the primer. The positioning of the template is critical for substrate

discrimination during the binding step, as we and others have shown that improper positioning of the templating base due to alteration of Arg-283, Met-282, or Thr-79 results in misinsertion by pol β (*22*, *31*, *35*). Because there is not a crystal structure of a ground-state binding complex, we are not certain of the positions of these amino acid residues or the DNA when the polymerase is in its "ground-state binding" form in the presence of dNTP substrate. However, some of these residues, including Met-282, Asn-279, and Asp-276 are not in their final positions to interact with the dNTP substrate when the enzyme is in an open form and must move up to 7 Å before being within proximity to the dNTP substrate (*36*). Other residues that form the binding pocket, such as Tyr-271 and Phe-272, are known to move about 5 Å during the closing of the polymerase. In addition, the DNA is repositioned upon the closing of the polymerase. This indicates that the nucleotide-binding pocket is not completely formed in the WT polymerase when it is in its open form. It is known that pol β does not strongly discriminate between nucleotide substrates during ground state binding, unlike other DNA polymerases, such as T7 or RB69 DNA polymerases, in which the residues that make up the nucleotide binding pocket are nearly in place during ground-state binding (*37*). Thus, the ability of a polymerase to discriminate against the incorrect substrate during ground-state binding may be associated with how well formed the nucleotide binding pocket is before the polymerase assumes a closed form.

Because Y265W is able to discriminate the dNTP substrate during ground-state binding on six-nucleotide gapped DNA somewhat better than WT, we suggest that the nucleotide binding pocket of this enzyme assumes a more defined and well-formed geometry during the dNTP binding step, compared to that of WT. This implies that both the character of the residue at position 265 and the type of DNA substrate bound by the polymerase can influence the geometry of the binding pocket. One mechanistic explanation for the role of residue 265 in forming the binding pocket is as follows. In WT pol β, Tyr-265 is part of a hydrophobic hinge region that is the pivot point for the enzyme as it moves from the open to the closed form (*36*). Tyr-265 appears to play a role in propping the hinge open when the enzyme is in the open form, and moves out of the way, so as to not prop the hinge open, when the polymerase assumes a closed form (*17*). A Trp residue at position 265 may not function in the same manner as a Tyr residue and may not serve in stabilizing the open form of the hinge as well as the Tyr. The difference in function could be due to the less polar nature of Trp verses Tyr, or the inability of Trp to form interactions of similar strength with some of the side chains of pol β with which Tyr interacts. If this were the case, the Y265W polymerase may assume a more closed conformation during ground-state binding than WT. The net result would be the formation of a more well-defined nucleotide-binding pocket in Y265W, due to its inability to function in stabilizing what may be the open form of the enzyme. A more well-defined binding pocket could provide a higher level of discrimination during ground-state binding.

The position and type of DNA substrate that is bound to the enzyme may also influence its conformation. The absence of five bases downstream of the primer may facilitate a repositioning of the DNA in the Y265W enzyme that is more permissive than single nucleotide gapped DNA in allowing the enzyme to assume a partially closed form, or a conformation that is more likely to bind the correct substrate. This conformation would presumably facilitate the formation of a well-defined binding pocket which would be more effective than a less well-formed pocket in excluding the incorrect dNTP substrate.

Mismatched Termini Are Extended by the Y265 Variants. In this study, we found that Y265W and Y265F are able to extend a mispaired primer terminus. The kinetic basis for the ability of the mutants to extend a mispaired primer terminus is nearly a complete lack of discrimination during ground state binding. Y265W extends the G:G mispair with a higher catalytic efficiency than Y265F, mainly due to the increased affinity of Y265W for the next correct nucleotide. The lack of discrimination during ground-state binding implies that the nucleotide binding pocket is not well defined by amino acid residues of pol β and by the DNA in the Y265W enzyme, and, to a lesser extent, in Y265F, specifically in the presence of a G:G mispair at the primer terminus. The fact that these enzymes have a higher level of discrimination at ground state binding in the absence of a mispair indicates that the binding pocket is well-defined during this step, and predominantly permits the correct nucleotide to enter. Thus, it follows that the Y265W and Y265F enzymes still maintain the potential to form a well-defined pocket, but only in the presence of a paired primer terminus. We suggest that in the presence of a mispaired primer-terminus the DNA that forms part of the binding pocket is repositioned such that it no longer acts as part of the walls that define the pocket. Here, the net result would be a more open pocket that would allow any dNTP substrate to bind to the enzyme, resulting in mispair extension. This indicates that the nature of the primer terminus influences nucleotide discrimination during ground-state binding. This affect could be direct, in that the primer terminus may form one of the walls of the nucleotide binding pocket. It could also be indirect, in that primer-terminus may influence the position of the templating base. For example, if the primer-terminus is matched, the templating base will be guided by Arg-283 and other residues of pol β to a position that is optimum for the accurate binding of substrate and catalysis of DNA synthesis. However, if the primer-terminus is mispaired, the templating base may be aberrantly positioned within in the binding pocket and permit an incorrect substrate to enter the pocket. In support of the hypothesis that the position of the DNA, especially the templating base, is important for discrimination during ground-state binding, we have shown that alteration of Thr-79 to Ser results in aberrant positioning of the templating base, resulting in a lack of nucleotide discrimination during ground-state binding (*35*). Y265W is most likely able to extend the G:G mispair with an incorrect substrate due to its more open pocket and its high affinity for dNTP substrate.

Y265F has the highest catalytic efficiency for extension of the T:G mispair, when compared to WT and Y265W, resulting mainly from a lack of discrimination during ground state binding. The Phe side chain has more similarities to Tyr than Trp, suggesting that the Y265F enzyme would behave more like WT in the extension of a T:G mispair. In support of this idea, WT appears to extend the T:G mispair slightly better than Y265W. We surmise that the position of the DNA and the templating base within the binding pocket is positioned much like it is in the presence of a correct base

pair, permitting extension of the T:G mispair. It is interesting that in the case of the T:G mispair, nucleotide discrimination increases during ground state binding for Y265W. The enhanced ability to discriminate during ground state binding was also observed during misinsertion (Table 2) and is most likely due to the reasons we discussed above. Briefly, the pocket of the Y265W enzyme assumes a more well-defined pocket, and the shape of the T:G may resemble that of a correctly paired terminus in this pocket.

Unlike Y265H (*17*), the Y265W and Y265F variants do not misincorporate nucleotides with a catalytic efficiency significantly different than pol β-WT. This suggests that the active site conformations of Y265W and Y265F are more similar to each other than to Y265H. Y265H losses its ability to discriminate correct from incorrect substrate during chemistry, whereas Y265F and Y265W maintain their abilities to discriminate at chemistry. This indicates that alignment during the transition state in the presence of an incorrectly paired nascent base pair is altered in Y265H, but not in Y265F or Y265W in comparison to WT. Therefore, substitution of Tyr-265 with other hydrophic residues results in a high fidelity enzyme from the standpoint of misincorporation, but an enzyme that can extend mispairs. This indicates that Tyr-265 is optimum, and that it most likely interacts with other amino acid residues of pol β to ensure accurate incorporation and to prevent mispair extension. These data demonstrate that the hinge region, and specifically the Tyr-265 pivot of the hinge, directly affect the conformation of the active site of pol β, and can have enormous impact upon the fidelity of pol β.

Normal and Mutator Polymerases May Coordinate to Produce Multiple Mutations. The results, especially for Y265W, support the idea of a mutator polymerase being able to generate multiple mutations during DNA synthesis in cancer cells (*2*, *38*). The biological framework of how this occurs is still a matter of conjecture. A possible scenario is that the wild-type pol β may misincorporate a nucleotide, which is followed by mispair extension by a mutant polymerase, such as Y265W, in cells which carry only one altered polymerase allele. This scenario seems plausible given the low fidelity of pol β (*39*). A similar model was proposed to bypass DNA lesions. In this model, it was suggested that pol ι incorporates opposite a lesion, while pol ζ extends the primer to bypass the damaged DNA, after pol ι-dependent insertion opposite the lesion (*40*). Thus, in theory, only one copy of a DNA polymerase gene would need to be mutated. The accumulated mutations present in the cancer cell genome may depend on the coordination of normal and mutant polymerases.

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