**Substrate-induced DNA Polymerase β Activation**

William A. Beard, David D. Shock, Vinod K. Batra, Rajendra Prasad, and Samuel H. Wilson

From the Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

**Background:** Highly conserved residues of DNA polymerase β alter their interactions as the enzyme transitions from open and closed conformations.

**Results:** Site-directed mutagenesis coupled with kinetic/structural characterization of key mutants defines the role(s) in enzyme activation.

**Conclusion:** These residues both hasten and deter correct and incorrect nucleotide insertion.

**Significance:** Conformational equilibrium(s) of the precatalytic ternary substrate complex influences the observed rate of nucleotide insertion.

DNA polymerases and substrates undergo conformational changes upon forming protein-ligand complexes. These conformational adjustments can hasten or deter DNA synthesis and influence substrate discrimination. From structural comparison of binary DNA and ternary DNA-dNTP complexes of DNA polymerase β, several side chains have been implicated in facilitating formation of an active ternary complex poised for chemistry. Site-directed mutagenesis of these highly conserved residues (Asp-192, Arg-258, Phe-272, Glu-295, and Tyr-296) and kinetic characterization provides insight into the role these residues play during correct and incorrect insertion as well as their role in conformational activation. The catalytic efficiencies for correct nucleotide insertion for alanine mutants were wild type ~ R258A > F272A > Y296A > E295A > D192A. Because the efficiencies for incorrect insertion were affected to about the same extent for each mutant, the effects on fidelity were modest (<5-fold). The R258A mutant exhibited an increase in the single-turnover rate of correct nucleotide insertion. This suggests that the wild-type Arg-258 side chain generates a population of non-productive ternary complexes. Structures of binary and ternary substrate complexes of the R258A mutant and a mutant associated with gastric carcinomas, E295K, provide molecular insight into intermediate structural conformations not appreciated previously. Although the R258A mutant crystal structures were similar to wild-type enzyme, the open ternary complex structure of E295K indicates that Arg-258 stabilizes a non-productive conformation of the primer terminus that would decrease catalysis. Significantly, the open E295K ternary complex binds two metal ions indicating that metal binding cannot overcome the modified interactions that have interrupted the closure of the N-subdomain.

DNA polymerases and their ligands (dNTPs, DNA, metals) undergo conformational adjustments upon complex formation. These conformational changes may be global in scope as exemplified by protein subdomain repositioning (1) or subtle, such as a change in protein side chain hydrogen bonding or metal coordination (2). Although great attention has focused on subdomain motions and their role in substrate discrimination, crystallographic studies have revealed that active site geometry is particularly sensitive to the identity of the conformational state of the complex (i.e. correct opposed to incorrect bound nucleotide) (3).

DNA polymerase β (pol β)² contributes two enzymatic activities during the repair of simple base lesions in genomic DNA; that is, template-directed DNA synthesis (nucleotidyltransferase) and deoxyribose 5′-phosphate removal (lyase) (4). These activities reside in separate domains: a 31-kDa polymerase domain and an 8-kDa amino-terminal lyase domain. The polymerase domain is structurally organized into functionally distinct subdomains referred to as DNA binding (D, residues 90–150), catalytic (C, 151–260), and nascent base pair binding (N, residues 261–335) subdomains (5). These are referred to as thumb, palm, and fingers subdomains, respectively, for right-handed polymerases that exhibit a non-homologous catalytic (palm) subdomain (6).

DNA binding (7), crystallography (2, 3, 8–10), NMR (11–13), and fluorescence studies (14–18) indicate that pol β and substrates undergo several conformational transitions upon binding substrates and metals during catalytic cycling. These transitions are believed to play a critical role during substrate discrimination, i.e. facilitate selecting right from wrong dNTPs. This is generally referred to as "induced fit" where binding of the correct nucleoside triphosphate results in an optimal alignment of catalytic residues that promotes binding and catalysis. In contrast, an incorrect dNTP leads to alternate protein/substrate conformations that discourage insertion. The kinetic result is that an incorrect substrate exhibits a lower apparent binding affinity (Kₐd) and decreased rate of nucleotide insertion (kₐp') relative to those for the correct incoming nucleotide.

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²The abbreviations used are: pol, DNA polymerase; PDB, Protein Data Bank; r.m.s.d., root mean square deviations; UMPNPP, 2′-deoxyuridine-5′-[(α,β)-imido]triphosphate.

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*This work was supported, in whole or in part, by National Institutes of Health Grants Z01-ES050158 and Z01-ES050161 (Intramural Research Program, NIEHS) and in association with the National Institutes of Health Grant U19CA105010. The atomic coordinates and structure factors (codes 4R63, 4R64, 4R65, and 4R66) have been deposited in the Protein Data Bank (http://wwpdb.org/).

¹To whom correspondence should be addressed: Laboratory of Structural Biology, NIEHS, National Institutes of Health, 111 T.W. Alexander Dr., P.O. Box 12233, MD F1-12, Research Triangle Park, NC 27709-2233. Tel.: 919-541-4701; Fax: 919-541-4724; E-mail: wilson5@niehs.nih.gov.
Crystallographic structures of binary DNA and ternary substrate complexes of pol β suggest that DNA and several protein side chains alter their position and/or interactions upon nucleotide binding (8) (Fig. 1). These interactions may provide a means by which the polymerase active site (i.e. metal binding ligands) detects whether the N-subdomain is able to form a stable closed complex. Alanine substitution for Arg-283 (>15 Å from the active site) results in a catalytically compromised enzyme that exhibits diminished fidelity (19–21). This loss in fidelity is completely due to the inability to insert the correct nucleotide. We now examine the role of several other residues that alter their interactions during formation of the closed ternary complex in modulating catalytic activation and fidelity.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure deoxynucleoside triphosphates, [γ-32P]ATP, and MicroSpin G-25 columns were from Amersham Biosciences-GE Healthcare.

Mutagenesis of the Human Pol β Gene—Oligonucleotide site-directed mutagenesis was performed using a procedure described previously (19). The codons for Asp-192, Arg-258, Phe-272, Glu-295, or Tyr-296 were altered to generate single alanine substitutions. Additionally, a double alanine mutant was generated at residues 258 and 272 (R258A/F272A), and an alternate lysine substitution was created at position 295 (E295K). To ensure that the resulting pol β gene contained the desired change(s), the entire coding sequence of the mutant was determined by Coomassie dye binding using purified pol β, single-turnover kinetic assays (enzyme/DNA = 10) were performed as outlined previously (25) employing a KinTek Model RQF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX). Typically, a solution of pol β (1 μM) was preincubated with single nucleotide-gapped DNA (100 nM). This solution was rapidly mixed (2-fold dilution) with various concentrations of dNTP/Mg2+.

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Protein Purification—Wild-type and mutant proteins were purified as described previously (23). Enzyme concentrations were determined by Coomassie dye binding using purified pol β as the standard. The concentration of purified pol β was determined by total amino acid analysis.

DNA Preparation—A 34-mer oligonucleotide DNA substrate containing a single nucleotide gap was prepared by annealing 3 gel-purified oligonucleotides (IDT, Coralville, IA) to create a single nucleotide gap at position 16. Each oligonucleotide was resuspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and the concentration was determined from their UV absorbance at 260 nm. The annealing reactions were carried out by incubating a solution of 10 μM primer with 12 μM concentrations of each of downstream and template oligonucleotides at 90–100 °C for 3 min followed by 30 min at 65 °C and then slow cooling to room temperature. The solution of the gapped DNA substrate was: primer, 5'-CTG CAG CTG ATG CGC-3'; downstream oligonucleotide, 5'-GTA CGG ATC CCC GGG TAC-3'; template, 3'-GAC GTC GAC TAC GCG XCA TGC CTA GGG GCC CAT G-5', where the X represents T or G. The primer was 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs), and radioactive ATP was removed with a MicroSpin G-25 column. The downstream oligonucleotide was synthesized with a 5'-phosphate.

Kinetic Assays—Steady-state kinetic parameters for single nucleotide gap-filling reactions were determined by initial velocity measurements as described previously (24). Unless noted otherwise, enzyme activities were determined using a standard reaction mixture containing 50 mM Tris-HCl, pH 7.4 (37 °C), 100 mM KCl, 5 mM MgCl2, and 200 mM single nucleotide-gapped DNA. Due to the lower stability of the R258A mutant at 37 °C, the reaction mixtures were supplemented with 50 μg/ml BSA. BSA does not influence the activity of the wild-type enzyme. Enzyme concentrations and reaction time intervals were chosen so that substrate depletion or product inhibition did not influence initial velocity measurements. Reactions were stopped with 20 μl of 0.5 M EDTA and mixed with an equal volume of formamide dye, and the products were separated on 12% denaturing polyacrylamide gels. The dried gels were analyzed using a PhosphorImager (Amersham Biosciences) to quantify product formation.

To directly measure the rate of the first insertion (kpol) and the apparent equilibrium nucleotide dissociation constant (Kpol), single-turnover kinetic assays (enzyme/DNA = 10) were performed as outlined previously (25) employing a KinTek Model RQF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX). Typically, a solution of pol β (1 μM) was preincubated with single nucleotide-gapped DNA (100 nM). This solution was rapidly mixed (2-fold dilution) with various concentrations of dNTP/Mg2+. Final conditions (pH, temperature) and salt concentrations were like those described for the steady-state assay. After various time periods, the reactions were stopped with 0.25 M EDTA, and the quenched samples were mixed with an equal volume of formamide dye. Products were separated and quantified as described above. Under these conditions the first-order rate constant of the exponential time-courses was dependent on the concentration of dNTP. A secondary plot of the concentration dependence of kobs was hyperbolic and fitted by a non-linear least-squares method to Equation 1 where kpol is the intrinsic rate constant for the step limiting the first insertion.

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k_{\text{obs}} = k_{\text{pol}}[\text{dNTP}]/(K_{d} + [\text{dNTP}])
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Crystallization of Mutant Pol β Substrate Complexes—The DNA substrate consisted of a 16-mer template, a complementary 10-mer primer strand, and a 5-mer downstream oligonucleotide. The annealed 10-mer primer creates a one-nucleotide gap with a templating A residue. The downstream oligonucleotide is 5'-phosphorylated. The template sequence was 5'-CCG ACA GCG CAT CAG C-3' (the underlined base is the coding nucleotide). Oligonucleotides were dissolved in 20 mM MgCl2 and 100 mM Tris-HCl, pH 7.5. Each set of template, primer, and downstream oligonucleotide was mixed in a 1:1:1 ratio and annealed using a PCR thermocycler by heating 10 min at 90 °C and cooling to 4 °C (1 °C/min) resulting in a 1 μM mixture of gapped duplex DNA. This solution was then mixed with an equal volume of mutant (R258A or E295K) pol β at 4 °C, and the mixture warmed to 35 °C and gradually cooled to 4 °C.

Pol β-DNA complexes were crystallized by sitting-drop vapor diffusion. The crystallization buffer was 16% PEG-3350, 350 mM sodium acetate, and 50 mM imidazole, pH 7.5. Drops were incubated at 18 °C and streak-seeded after 1 day. Crystals grew in ~2–4 days after seeding. The ternary complex was obtained by soaking crystals of binary 1-nucleotide-gapped
DNA complexes in artificial mother liquor with 100 mM MgCl₂ or 50 mM MnCl₂ and with 2 mM dUMPNPP, 20% PEG-3350, and 12% ethylene glycol and then flash-frozen to 100 K in a nitrogen stream. All crystals belong to the space group P₂₁₂₁.

Data Collection and Structure Determination—X-ray crystal diffraction data were collected on a Saturn 92 CCD detector system mounted on a MicroMax-007HF (Rigaku Corp.) rotating anode generator. Data were integrated and reduced for structure refinement with HKL2000 software (26).

Structures were determined by molecular replacement with previously determined structures of pol β complexed with a one-nucleotide gapped DNA (binary complex, PDB ID 3ISB) (27) or a ternary complex with an incoming dUMPNPP (ternary complex, PDB ID 2FMS) (9). The crystal structures have similar lattices and are sufficiently isomorphous to determine the molecular replacement model using PHENIX (28). Further refinement and model building were carried out using O (29). The molecular graphic images were prepared in Chimera (30).

Accession Codes—Protein Data Bank coordinates and structure factors for the pol R258A pol β/DNA binary and ternary (+dUMPNPP) and E295K pol β/DNA binary and ternary (+dUMPNPP) complexes have been deposited with accession codes 4R63, 4R65, 4R64, and 4R66, respectively.

RESULTS

Site-directed Mutagenesis—Comparing crystallographic structures of different liganded states of pol β indicate that DNA around the active site and several side chains of the C- and N-subdomains alter their interactions upon going from an open binary DNA complex to a closed ternary substrate complex (8, 31) (Fig. 1). These altered interactions result from repositioning of the N-subdomain upon dNTP binding. Specifically, when the N-subdomain is in the open “inactive” conformation, Asp-192 forms a salt bridge with Arg-258 thereby diverting a critical metal coordinating ligand (Fig. 1B). A conservative glutamate substitution at Asp-192 (D192E) results in a dramatic loss of catalytic activity highlighting the critical role of this residue (32). Asp-190 and Asp-192 coordinate both Mg²⁺ ions necessary for catalysis. In the closed conformation, the phenylalanine ring of residue 272 is positioned between Asp-192 and Arg-258, thereby insulating these residues. Asp-192 and Arg-258 have rotated away from one another to coordinate active site metals or form hydrogen bonds with Glu-295 and Tyr-296, respectively. Interestingly, a lysine mutant at residue 295 has been detected in some human gastric cancers and a role in modulating base excision repair suggested (33). Molecular modeling of the N-subdomain movement has suggested that side-chain motions do not occur in a concerted manner but follow a sequential path (34–36). To probe the functional significance of these residues, alanine has been substituted for Asp-192 (D192A), Arg-258 (R258A), Phe-272 (F272A), Glu-295 (E295A), or Tyr-296 (Y296A). Additionally, lysine was substituted for Glu-295 (E295K) to address the functional consequences that may lead to the observed biological repercussions (33). The mutant proteins were expressed in E. coli and purified. The purified mutant pol β proteins were >95% homogeneous and devoid of contaminating exonuclease activity (data not shown). The catalytic consequences of these protein modifications were assessed.

Single Nucleotide Gap-filling DNA Synthesis—To analyze the effect of the altered side chain on DNA synthesis, we determined steady-state kinetic parameters for insertion of a correct nucleotide (dATP or dCTP) into a single nucleotide-gapped heteropolymeric DNA substrate (templating thymine or guanine, respectively) (Table 1; Fig. 2). For most mutants, k_{cat} for correct insertion was minimally affected by alanine substitution. The R258A mutant exhibited a modest increase in k_{cat} for correct insertion was minimally affected by alanine substitution. The R258A mutant exhibited a modest increase in the respective turnover numbers for correct insertion. However, it
should be noted that the steady-state rate of correct nucleotide insertion is partially limited by at least two kinetic steps, chemistry and product dissociation (25). In contrast, $k_{\text{cat}}$ is significantly decreased for incorrect insertion relative to wild-type enzyme (Table 2). It is generally believed that the rate of incorrect nucleotide insertion is limited by chemistry so that $K_{i,m}$ represents the apparent equilibrium dissociation constant, $K_p$, for the incorrect nucleotide.

The catalytic efficiency of correct insertion for the E295A mutant was reduced by $>10,000$-fold relative to wild-type enzyme (Table 2).

### Table 1: Steady-state kinetic summary for single nucleotide gap-filling DNA substrates

| Enzyme | Template dNTP | $k_{\text{cat}}$ | $K_{m,dNTP}$ | $k_{\text{cat}}/K_{m}$ |
|--------|---------------|-----------------|--------------|-----------------|
| Wild type | dT-dATP | 90 (10) | 1.1 (0.04) | 8200 (1000) |
| | dt-dGTP | 9 (1) | 480 (110) | 1.9 (0.5) |
| | dG-dCTP | 58 (3) | 0.40 (0.10) | 14500 (2700) |
| | dG-dTTP | 4.1 (0.3) | 1300 (170) | 0.31 (0.05) |
| D192A | dG-dCTP | I* | I* | I* |
| D192E | dG-dCTP | ND | ND | ND |
| R258A | dT-dATP | 180 (20) | 7.9 (0.4) | 2300 (300) |
| | dt-dGTP | 4 (0.9) | 1635 (98) | 0.24 (0.06) |
| | dG-dCTP | 120 (6) | 3.2 (0.9) | 3750 (1070) |
| | dG-dTTP | 2.1 (0.1) | 1150 (100) | 0.18 (0.02) |
| F272A | dT-dATP | 20 (1) | 7.2 (1.6) | 280 (60) |
| | dt-dGTP | 0.22 (0.06) | 1200 (40) | 0.018 (0.005) |
| | dG-dCTP | 6.5 (0.2) | 1.3 (0.2) | 500 (80) |
| | dG-dTTP | 0.076 (0.001) | 1448 (46) | 0.0052 (0.0002) |
| E295A | dT-dATP | 15.0 (1.0) | 70 (6) | 21 (2) |
| | dt-dGTP | ND | ND | ND |
| | dG-dCTP | 14.2 (0.7) | 20 (2) | 71 (8) |
| | dG-dTTP | ND | ND | ND |
| E295K | dG-dCTP | ND | ND | ND |
| Y296A | dT-dATP | 86 (20) | 75 (8) | 115 (30) |
| | dt-dGTP | 0.05 (0.01) | 450 (50) | 0.011 (0.0002) |
| | dG-dCTP | 66 (8) | 29 (3) | 230 (40) |
| | dG-dTTP | 0.049 (0.003) | 1023 (120) | 0.0048 (0.0006) |

* Under steady-state conditions, reliable kinetic parameters activity could not be determined.

### Figure 2. Effect of alanine substitution for Arg-258, Phe-272, Glu-295, and Tyr-296 on catalytic efficiency and substrate discrimination.

A, a discrimination (57) plot for 1-nucleotide gap filling (templating dT). The catalytic efficiencies ($k_{\text{cat}}/K_m$, $\mu M^{-1} s^{-1}$) for correct (green) dATP insertion are compared with those for incorrect (red) dGTP insertion. The distance between these points on the ordinate is a measure of substrate discrimination. B, a discrimination plot for one-nucleotide gap filling (templating dG). The catalytic efficiencies ($k_{\text{cat}}/K_m$, $\mu M^{-1} s^{-1}$) for correct (green) dCTP insertion are compared with those for incorrect (red) dTTP insertion. C, the relative (mutant/wild-type) fidelity (efficiency-correct/efficiency-incorrect) of the mutants was calculated from the data tabulated in Table 1. The bars above each point represent an increase in fidelity, and those below each point exhibit a decrease in fidelity.
change in the rate-limiting step for correct nucleotide insertion when analyzing a mutant enzyme. For wild-type pol β, catalytic cycling for correct nucleotide DNA synthesis is limited by insertion ($k_{\text{pol}}$) and product release ($k_{\text{off,DNA}}$) (25). This precludes direct determination of the intrinsic rate constant for insertion by presteady-state methods as the observed exponential rate constant of the burst phase includes a contribution from product release. Alternatively, a single-turnover analysis ($E \gg \text{DNA}$) can be used to eliminate catalytic cycling and directly measure $k_{\text{pol}}$ and $K_{d,\text{dNTP}}$ (37). Under this condition, the single-exponential time courses for correct nucleotide gap-filling DNA synthesis is dependent on dATP concentration (Fig. 3A). A secondary plot of the nucleotide concentration dependence of $k_{\text{obs}}$ provides estimates of $k_{\text{pol}}$ and $K_{d,\text{dNTP}}$ (Equation 1; Fig. 3B, Table 2).

Because Arg-258 forms a salt bridge with Asp-192 in the binary DNA complex, this acidic side chain is not available to provide the necessary ligands for the active site metals needed for catalytic activation (Fig. 1B). Modeling of the N-subdomain closing motion upon binding a correct nucleotide identified several conformational transitions that may occur as Arg-258 releases Asp-192 and forms hydrogen bonds with Glu-295 and Tyr-296 (36). Accordingly, the Arg-258/Asp-192 interaction represents a key barrier that must be overcome to permit formation of an active catalytic site. If the events required for this enzyme activation pathway are kinetically or thermodynamically significant, alanine substitution for Arg-258 could lower the barrier for catalytic activation and/or might augment the population of activated complex (see “Discussion”), resulting in an increase in the observed (i.e. measured) rate of nucleotide insertion.

In the presence of a saturating concentration of the correct nucleotide (dATP), the single-exponential time course provides a measure of $k_{\text{pol}}$ (Fig. 4A). Although the observed rate of correct nucleotide insertion was dependent on the identity of the incoming nucleotide, the rate was significantly more rapid for the R258A mutant than wild-type enzyme (Fig. 4). From the nucleotide concentration dependence of the observed rate constants for these single-exponential time courses, the binding affinity for the incoming nucleotide was 5-fold lower for the R258A mutant relative to wild type (Table 2). This results in a 2.3-fold loss in the specificity constant ($k_{\text{pol}}/K_a$), similar to that determined by the steady-state kinetic approach ($k_{\text{cat}}/K_m$, Table 1).

Single-turnover Kinetic Analysis of F272A—The decrease in the efficiency of correct nucleotide insertion observed for the F272A mutant (Table 1) suggests that nucleotide insertion rather than product release is rate determining during steady-state turnover. This was verified by directly measuring $k_{\text{pol}}$ employing single-turnover conditions as described above. The time course indicates that dATP insertion occurs at a similar rate as that observed for the steady-state rate (Fig. 5 and Table 1).

**TABLE 2**

| Enzyme     | Template-dNTP | $k_{\text{pol}}$ | $K_{d,\text{dNTP}}$ | $k_{\text{pol}}/K_{d,\text{dNTP}}$ |
|------------|---------------|------------------|---------------------|-----------------------------------|
| Wild type  | dT-dATP       | 7.6 (0.3)        | 4.6 (0.7)           | 1.62 × 10⁻⁴ μM⁻¹ s⁻¹              |
|            | dG-dCTP       | 3.3 (0.2)        | 1.6 (0.4)           | 20,625 (5,300)                    |
| D192E      | dG-dCTP       | 2.0 × 10⁻⁴ (6 × 10⁻⁶)² |                      |                                   |
| R258A      | dT-dATP       | 18.0 (0.3)       | 25 (2)              | 7,200 (590)                       |
|            | dG-dCTP       | 7.2 (0.2)        | 8.4 (0.8)           | 8,570 (850)                       |
| E295A      | dG-dTTP       | 9.55 × 10⁻³ (1.2 × 10⁻⁵) | 550 (30)            | 0.0017 (0.0002)                   |
|            | dT-dGTP       | 1.58 × 10⁻³ (2.0 × 10⁻⁵) | 215 (5)             | 0.0073 (0.0002)                   |
| E295K      | dG-dCTP       | 3.1 × 10⁻³ (2.0 × 10⁻⁴) | 5 (2)               | 6.2 (2.5)                        |
|            | dG-dTTP       | 1.35 × 10⁻³ (1.6 × 10⁻⁴) | 250 (90)            | 0.00054 (0.0002)                 |

* Template nucleotide-incoming nucleoside triphosphate.
* Observed rate constant at 250 μM dCTP.

**FIGURE 3.** dATP concentration dependence of single nucleotide incorporation by wild-type pol β. A, wild-type enzyme (1 μM) was preincubated with DNA (pol/DNA = 10) and rapidly mixed with 0.2 ( ), 1.1 ( ), 2 ( ), 11 ( ), 20 ( ), or 110 μM ( ) dATP. Reactions were quenched at the indicated times, and the products were isolated and quantified as described under “Experimental Procedures.” The solid lines represent the best fit of the data to a rising exponential, B, a secondary plot of the dATP concentration dependence of the observed first-order rate constants measured in panel A. The data were fitted to a hyperbola (Equation 1) to yield a $K_{d,\text{dATP}}$ of 4.6 μM and $k_{\text{pol}}$ of 7.6 s⁻¹ (Table 2).
These results with the single alanine mutants indicate that Arg-258 modulates wild-type insertion negatively (decreases insertion), whereas Phe-272 facilitates insertion. If the only role of Phe-272 is to insulate Arg-258 and Asp-192, then eliminating the Arg-258 and Asp-192 interaction through alanine substitution for Arg-258 should rescue the loss in activity observed with alanine substitution for Phe-272. However, if Phe-272 has a different or an additional role, then the double mutant will behave like the F272A mutant. The single-turnover time course performed with saturating dATP indicates that $k_{\text{pol}}$ for the double alanine mutant R258A/F272A is identical to that for single F272A mutant (Fig. 5). Thus, Phe-272 provides an additional role distinct from insulating Asp-192 and Arg-258.

**Single-turnover Kinetic Analysis of E295K**—The poor insertion efficiency of the gastric cancer-associated E295K mutant precluded a steady-state kinetic analysis. Using conditions adequate for single-turnover analyses that utilize high enzyme concentrations, insertion ($k_{\text{pol}}$) and dNTP binding ($K_{d,\text{dNTP}}$) can be assessed. Under these conditions where catalytic cycling does not occur, events at the polymerase active site are measured. The 2400-fold loss in catalytic efficiency ($k_{\text{pol}}/K_{d}$) with the lysine mutant is completely due to the inability to insert the correct nucleotide ($k_{\text{pol}}$) as the affinity for the correct nucleotide is not diminished (Table 2). The specificity constant for misinsertion of dTTP opposite a templating guanine was 11,500-fold lower than for dCTP insertion. This suggests that the fidelity of this mutant is 4-fold lower than wild-type enzyme in this DNA sequence context. The poor insertion efficiency for this mutant enzyme precludes it from posing a direct mutagenic threat to the genome but could pose an indirect threat as a trans-dominant inhibitor of base excision DNA repair.

**Structural Characterization of R258A and E295K**—Crystallographic structures of binary single nucleotide-gapped DNA and ternary (dUMPNPP) substrate complexes were determined for two mutants (i.e. R258A and E295K) that would be expected to alter the dynamics of the residues participating in hydrogen bonding during catalytic cycling (Table 3). The global conformation of the binary and ternary complex structures of the R258A mutant is nearly identical to that of wild-type enzyme (r.m.s.d. for all C$_\text{α}$ 0.16 and 0.20 Å with the open binary and closed ternary wild-type complexes). Like wild-type enzyme, the global conformation of the binary DNA complex is open, whereas the ternary complex is in a closed conformation (Fig. 6A). Except that the arginine has been replaced with alanine, the other signaling residues superimpose with their wild-type counterparts (Fig. 6B).
In contrast to the lack of significant structural perturbation observed with the R258A mutant, the E295K mutant exhibited significant structural differences in the ternary substrate complex compared with wild-type enzyme. Although the open binary complex was similar to wild-type enzyme, the ternary substrate complex remained in the open conformation upon binding a complementary non-hydrolysable incoming nucleotide (Fig. 7A). In this case, two manganese ions coordinate the triphosphate moiety of the incoming nucleotide, whereas the Watson-Crick edge of the uracil base hydrogen bonds with the templating adenine. The nascent base pair exhibits a severe buckling (Fig. 7B). As a result, O3’ of the primer terminus hydrogen bonds with Arg-258, effectively removing this essential catalytic atom from the active site (Fig. 7C).

**DISCUSSION**

**Residue Conservation**—The residues examined here were based on their proposed role in protein conformational adjustments as a result of substrate binding. Consequently, their functional role could be in substrate binding, substrate discrimination, and/or catalysis. The high conservation exhibited by these residues when compared with all members of the DNA polymerase X-family is consistent with a critical functional role for these residues (Table 4). Interestingly, these residues do not appear to be highly conserved when compared with the other three human X-family enzymes. This later observation is consistent with the divergent functional roles these enzymes have in the cell (38). Likewise, these other human enzymes (pol λ, pol μ, and terminal deoxynucleotidyltransferase) do not exhibit an open to closed subdomain repositioning when they form a ternary substrate complex (39–41).

Not surprisingly, the strongest kinetic consequences were observed with the two most conserved residues, Asp-192 and Arg-283. Alanine substitution for these residues effectively eliminates activity. Whereas activity is not measurable for the metal binding ligand D192A (Table 1), catalytic efficiency is decreased >104-fold for the alanine mutant of Arg-283 (21). The high conservation of these residues among all X-family DNA polymerases indicates that they have an essential role in addition to their role in conformational changes associated with subdomain motions.

**Structural Insights**—The high resolution crystallographic structures of the binary and ternary precatalytic complexes of the R258A mutant are very similar to those of the wild-type enzyme. As illustrated in Fig. 6B, the side-chain conformations of the residues that propagate a hydrogen bonding cascade in the open and closed polymerase conformations are similar to that observed with the corresponding binary and ternary complex structures of wild-type enzyme. Removing the potential for a salt bridge with Asp-192 or Glu-295 apparently does not have a strong influence on the position of these side chains in the

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**TABLE 3**

Crystallographic data and refinement statistics

| Complex | Binary | Ternary |
|---------|--------|--------|
| PDB code | 4R63 4R64 | 4R65 4R66 |
| Data collection |
| a (Å) | 54.4 | 53.5 | 50.8 | 55.0 |
| b (Å) | 79.3 | 79.0 | 79.9 | 79.2 |
| c (Å) | 54.8 | 54.5 | 55.5 | 54.9 |
| β (%) | 105.8 | 106.1 | 107.3 | 92.9 |
| Resolution range (Å) | 50-1.85 (1.92-1.85) | 50-2.20 (2.28-2.20) | 50-1.95 (2.02-1.95) | 50-2.25 (2.33-2.25) |
| Rmerge (%) | 9.9 (48.4) | 5.8 (28.9) | 7.5 (39.2) | 8.9 (25.2) |
| Completeness (%) | 99.7 (99.0) | 95.9 (93.7) | 91.6 (63.7) | 98.7 (89.9) |
| I/σ(I) | 13.8 (2.8) | 16.6 (3.8) | 17.4 (2.6) | 14.7 (3.3) |
| No. of observed reflections | 233,174 | 79,350 | 96,563 | 79,343 |
| No. of unique reflections | 38,158 (3,750) | 21,541 (2,114) | 28,340 (1,958) | 22,091 (2,002) |
| Wavelength (Å) | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Refinement |
| r.m.s.d. |
| Bond lengths (Å) | 0.007 | 0.008 | 0.007 | 0.008 |
| Bond angles (°) | 1.15 | 1.05 | 1.14 | 1.12 |
| Rwork (%) | 23.2 | 24.3 | 22.2 | 24.8 |
| Rfree (%) | 18.0 | 19.0 | 18.7 | 18.1 |
| Average B factor (Å²) | 21.2 | 40.5 | 23.6 | 31.7 |
| Protein | 21.4 | 34.7 | 31.4 | 27.2 |
| DNA | 28.7 | 42.2 | 30.5 | 34.1 |
| dUMPNNPP | NA | NA | 15.5 | 39.9 |
| Ramachandran analysis[a] |
| Allowed (%) | 97.5 | 95.4 | 99.1 | 96.3 |
| Favorable (%) | 100 | 99.1 | 100 | 98.8 |

[a] The binary single nucleotide-gapped DNA complexes have deoxyadenosine in the coding-unpaired position of the template strand. The ternary complex includes the non-hydrolysable dUMPNNPP analog.

[b] Highest resolution shell.

[c] Rmerge = 100Σ||Ih,i|−|Ih,i||/100Σ|Ih,i|, where Ih,i is the mean intensity of symmetry-related reflections Ih,i.

[d] Numbers in parentheses refer to the highest resolution shell of data (10%).

[e] Rwork = 100Σ||Fc|−|Fo||/Σ|Fc|.

[f] Rfree is partially limited by at least two kinetic steps for a 5% subset of reflections withheld from refinement.

[g] NA, not applicable.

[h] Data were determined by MolProbity (58).
binary or ternary complexes. Because these structures represent static endpoints, intermediate events must be isolated or trapped with mutant enzymes, substrate analogs, or through computational studies.

In contrast to the structures of the R258A mutant, the ternary complex structure of the lysine mutant of Glu-295 is unique. The E295K variant of pol β has been identified in gastric (33) and colon carcinomas (42) and shown to have low activity (Table 2) (43, 44). Although the overall conformation is similar to that of the open ternary complex observed previously for the R283K mutant with a correct incoming nucleotide (2), the structure of the ternary complex of E295K exhibits two Mn$^{2+}$ ions that coordinate the phosphate moiety of the incoming dUMPNPP (Fig. 7B). In contrast, binding of the second Mn$^{2+}$ ion to the R283K mutant resulted in a closed ternary complex. Importantly, the trapped open complex of the E295K mutant indicates that Arg-258 can hydrogen bond with O3’ of the primer terminus that has moved to a catalytically inactive position (Fig. 7C). A similar open ternary complex of wild-type enzyme with two manganese ions has been reported for pol β inserting dCMPNPP opposite 2’-fluoro-N7-methylguanine (45). However, in this case, O3’ of the primer terminus coordinates the catalytic metal rather than interacting with Arg-258. A structure of an open mismatch E295K ternary complex structure has been reported by Eckenroth et al. (43) indicating that Arg-258 and Asp-256 can interact with O3’ of the primer terminus through a water-mediated hydrogen bond. This results in an inactive conformation as O3’ is misaligned and Asp-256 cannot coordinate the catalytic metal.

The structural results support a model where both Arg-258 and Glu-295 influence active site conformational equilibria between active and inactive conformations. Glu-295 stabilizes the closed active complex upon forming a ternary substrate complex, and altering this interaction(s) can result in an inactive conformation(s); that is, open N-subdomain and displaced primer terminus stabilized by Arg-258. Consequently, activity of this mutant is severely decreased. In contrast, Arg-258 interacts with a metal binding ligand (Asp-192) in the binary DNA complex and is observed to stabilize an inactive complex in the ternary complex of the E295K mutant. Accordingly, removal of these possible inactivating interactions by alanine substitution potentially enhances the population of active ternary substrate complex that would result in the observed apparent increase in insertion (Fig. 4). An apparent increase in the activity of the R258A mutant on a homopolymeric template-primer system had also been reported (46).

Conformational Activation and Deactivation—Because many DNA polymerases appear to utilize substrate-induced conformational changes to align catalytic groups, an induced fit mechanism has been proposed to describe substrate specificity. Good substrates optimize the active site by aligning catalytic atoms, whereas poor substrates deter catalysis through the misalignment of reactive atoms. Although protein and substrate conformational adjustments can be rapid, the equilibrium with non-catalytic complexes can influence polymerase fidelity (47, 48).

Because single nucleotide insertion appears to be limited by a chemical step rather than conformational adjustments (16, 49), the increased rate of insertion exhibited by the R258A mutant must be due to a change in the thermodynamic equilibrium with non-productive complexes rather than a kinetic change in the rate-limiting step. Scheme 1 illustrates structural adjustments that occur in the enzyme and/or substrates of the ternary substrate complex. In this oversimplified scheme, $k_{\text{DNA}^E\text{dNTP}}$ represents the active ternary substrate complex that irreversibly forms product ($k_{\text{pol}}$). F represents an inactive enzyme conformation (e.g. open conformation), whereas the substrates may be bound in a catalytically active or inactive state (denoted by their subscript or superscript position; the inactive forms are highlighted in red). In this scenario the measured single-turnover rate of nucleotide insertion is $k_{\text{obs}} = k_{\text{pol}}(\text{DNA}^E\text{dNTP})/E_T$ where $E_T$ represents the sum of all ternary substrate complexes. Accordingly, the measured rate constant is not only dependent on the intrinsic rate constant for nucleotide insertion ($k_{\text{pol}}$) but also on the relative concentration of active ternary substrate.
complex. If this complex is in equilibrium with non-productive complexes, then the measured rate underestimates the true intrinsic rate constant. Although the rate-limiting step for the forward reaction is the chemical step, altering the conformational equilibrium with alternate non- or less-productive forms of the ternary substrate complex would decrease the observed rate.

The inactive ternary complex $\text{DNA}^{*}E^{-}\text{dNTP}$ (complex i) represents a complex where the DNA substrate is not correctly aligned (e.g.
primer terminus in the incoming nucleotide binding pocket) (13), DNA $F_{\text{dNTP}}$ (complex ii) represents a protein conformational defect (e.g. open conformation) (2), and DNA $E_{\text{dNTP}}$ (complex iii) represents a complex where the incoming dNTP is not positioned correctly (e.g. poor metal coordination (Fig. 7B)) (2). In reality, these substrate and protein irregularities would be expected to impact one another (i.e. protein conformation influence substrate alignment). However, it serves to illustrate the simple idea that enzyme-substrate complexes are not a simple homogeneous species. The open and closed forms of many DNA polymerases have long been recognized (1), and single-molecule studies have revealed a spectrum of polymerase populations in varying states of “openness” (50). Using a fluorescence probe attached to the N-subdomain (i.e. fingers of A-family DNA polymerases), Tsai and Johnson (48) identified a unique substrate complex conformation induced by binding an incorrect nucleotide they termed “misaligned.” Thus, DNA polymerases can prevent misinsertion through inducing an inactive complex where the templating nucleotide moves upstream vacating the coding position and repositioning the primer terminus away from the active site, effectively deterring misinsertion (10). For pol $\beta$, other conformational changes that occur before chemistry have been identified through protein (18) and DNA fluorescence changes (17, 51). In these later studies, the R258A alanine substitution for Arg-258 results in less inactive ternary complex where the Phe-272 side chain insulates the catalytic site from the incoming nucleotide, replacing the large hydrophobic surface with a single methyl group would be expected to alter active site interactions in the vicinity of the chemical reaction. Consistent with this interpretation, previous characterization of a leucine substitution at residue 272 did not observe a loss in the insertion rate of a correct nucleotide, indicating that the longer aliphatic side chain did not dramatically distort the active site (56). Because Phe-272 is also situated on $\alpha$-helix M of the N-subdomain, it undergoes a small rotation as pol $\beta$ transitions between open and closed states (Fig. 1B) (4). Thus, Phe-272 plays unique roles depending on the conformational/catalytic state of the enzyme. It appears to be involved in (1) the open/closed enzyme conformational transition, (2) precise positioning of the incoming nucleotide, and (3) insulating Asp-192 and Arg-258 from one another.

Lys-280 also appears to play disparate roles in correct nucleotide insertion depending on the identity of the templating nucleotide. Whereas this side chain contributes key interactions with templating purines, these interactions are far less important with templating pyrimidines (5). Accordingly, it should not be surprising to discover that an enzyme side chain has multi-faceted roles during DNA synthesis that depend on the identity of the incoming nucleotide and DNA sequence.

### Acknowledgments

We thank Drs. K. Bebenek and B. D. Freudenthal for critical reading of the manuscript and valuable discussions. Molecular graphics images were produced using the Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health Grant P41 GM-103311).
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REFERENCES
1. Doublé, S., Sawaya, M. R., and Ellenberger, T. (1999) An open and closed case for all polymerases. Structure 7, R31–R35
2. Freudenthal, B. D., Beard, W. A., and Wilson, S. H. (2012) Structures of dNTP intermediate states during DNA polymerase active site assembly. Structure 20, 1829–1837
3. Freudenthal, B. D., Beard, W. A., Shock, D. D., and Wilson, S. H. (2013) Observing a DNA polymerase choose right from wrong. Cell 154, 157–168
4. Beard, W. A., and Wilson, S. H. (2014) Structure and mechanism of DNA polymerase β. Biochemistry 53, 2768–2780
5. Beard, W. A., Shock, D. D., Yang, X.-P., DeLauder, S. F., and Wilson, S. H. (2002) Loss of DNA polymerase β stacking interactions with templating purines, but not pyrimidines, alters catalytic efficiency and fidelity. J. Biol. Chem. 277, 8235–8242
6. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Crystal structure of rat DNA polymerase β: evidence for a common polymerase mechanism. Science 264, 1930–1935
7. Beard, W. A., Shock, D. D., and Wilson, S. H. (2004) Influence of DNA structure on DNA polymerase β active site function: extension of mutagenic DNA intermediates. J. Biol. Chem. 279, 31921–31929
8. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: evidence for an induced fit mechanism. Biochemistry 36, 11205–11215
9. Batra, V. K., Beard, W. A., Shock, D. D., Krahn, J. M., Pedersen, L. C., and Wilson, S. H. (2006) Magnesium induced assembly of a complete DNA polymerase catalytic complex. Structure 14, 757–766
10. Batra, V. K., Beard, W. A., Shock, D. D., Pedersen, L. C., and Wilson, S. H. (2008) Structures of DNA polymerase β with active site mismatches suggest a transient abasic site intermediate during misincorporation. Mol. Cell 30, 315–324
11. Bose-Basu, B., DeRose, E. F., Kirby, T. W., Mueller, G. A., Beard, W. A., Wilson, S. H., and London, R. E. (2004) Dynamic characterization of a DNA repair enzyme: NMR studies of [methyl-13C]methionine-labeled DNA polymerase β. Biochemistry 43, 8911–8922
12. Berlow, R. B., Swain, M., Dalal, S., Sweasy, J. B., and Loria, J. P. (2012) Substrate-dependent millisecond domain motions in DNA polymerase β. J. Mol. Biol. 419, 171–182
13. Kiry, T. W., DeRose, E. F., Cavanaugh, N. A., Beard, W. A., Shock, D. D., Mueller, G. A., Wilson, S. H., and London, R. E. (2012) Metal-induced translocation leads to DNA polymerase conformational activation. Nucleic Acids Res. 40, 2974–2983
14. Dunlap, C. A., and Tsai, M.-D. (2002) Use of 2-aminopurine and tryptophan fluorescence as probes in kinetic analyses of DNA polymerase β. Biochemistry 41, 11226–11235
15. Date, T., Yamamoto, S., Tanihara, K., Nishimoto, Y., and Matsukage, A. (1991) Aspartic acid residues at positions 190 and 192 of rat polymerase β are involved in primer binding. Biochemistry 30, 5286–5292
16. Iwana, A., Ouchida, M., Miyazaki, K., Hori, K., and Mukai, T. (1999) Functional mutation of DNA polymerase β found in human gastric cancer: inability of the base excision repair in vitro. Mutat. Res. 435, 121–128
17. Lang, Y., Beard, W. A., Wilson, S. H., Broyde, S., and Schlick, T. (2002) Polymerase β simulations suggest that Arg-258 rotation is a slow step rather than large subdomain motions per se. J. Mol. Biol. 317, 651–671
18. Lang, Y., Beard, W. A., Wilson, S. H., Broyde, S., and Schlick, T. (2004) Highly organized but pliant active site of DNA polymerase β: compensatory mechanisms in mutant enzymes revealed by dynamics simulations and energy analyses. Biophys. J. 86, 3392–3408
19. Radhakrishnan, R., Arora, K., Wang, Y., Beard, W. A., Wilson, S. H., and Schlick, T. (2006) Regulation of DNA repair fidelity by molecular checkpoints: “gates” in DNA polymerase β’s substrate selection. Biochemistry 45, 15142–15156
20. Beard, W. A., and Wilson, S. H. (2006) Structure and mechanism of DNA polymerase β. Chem. Rev. 106, 361–382
21. Moon, A. F., Garcia-Diaz, M., Batra, V. K., Beard, W. A., Broyde, K., Kunkel, T. A., Wilson, S. H., and Pedersen, L. C. (2007) The X family portrait: structural insights into biochemical functions of X family polymerases. DNA Repair 6, 1709–1725
22. Moon, A. F., Pryor, J. M., Ramsden, D. A., Kunkel, T. A., Broyde, K., and Pedersen, L. C. (2014) Sustained active site rigidity during synthesis by human DNA polymerase μ. Nat. Struct. Mol. Biol. 21, 253–260
23. Garcia-Diaz, M., Broyde, K., Kunkel, T. A., and Pedersen, L. C. (2005) A closed conformation for the Pol μ catalytic cycle. Nat. Struct. Mol. Biol. 12, 97–98
41. Gouge, J., Rosario, S., Romain, F., Beguin, P., and Delarue, M. (2013) Structures of intermediates along the catalytic cycle of terminal deoxynucleotidyltransferase: dynamical aspects of the two-metal ion mechanism. J. Mol. Biol. 425, 4334–4352.

42. Donigan, K. A., Sun, K.-W., Nemec, A. A., Murphy, D. L., Cong, X., Northrup, V., Zelterman, D., and Sweasy, J. B. (2012) Human POLB gene is mutated in high percentage of colorectal tumors. J. Biol. Chem. 287, 23830–23839.

43. Eckenroth, B. E., Towle-Weicksel, J. B., Sweasy, J. B., and Doublée, S. (2013) The E295K cancer variant of human polymerase δ9252 favors the mismatch conformational pathway during nucleotide selection. J. Biol. Chem. 288, 34850–34860.

44. Lang, T., Dalal, S., Chikova, A., DiMaio, D., and Sweasy, J. B. (2007) The E295K DNA polymerase δ9252 gastric cancer-associated variant interferes with base excision repair and induces cellular transformation. Mol. Cell. Biol. 27, 5587–5596.

45. Koag, M.-C., Kou, Y., Ouzon-Shubeita, H., and Lee, S. (2014) Transition-state destabilization reveals how human DNA polymerase δ9252 proceeds across the chemically unstable lesion N7-methylguanine. Nucleic Acids Res. 42, 8755–8766.

46. Menge, K. L., Hostomsky, Z., Nodes, B. R., Hudson, G. O., Rahmati, S., Moomaw, E. W., Almassy, R. J., and Hostomska, Z. (1995) Structure-function analysis of the mammalian DNA polymerase δ9252 active site: role of aspartic acid 256, arginine 254, and arginine 258 in nucleotidyl transfer. Biochemistry 34, 15934–15942.

47. Johnson, K. A. (2008) Role of induced fit in enzyme specificity: a molecular forward/reverse switch. J. Biol. Chem. 283, 26297–26301.

48. Tsai, Y.-C., and Johnson, K. A. (2006) A new paradigm for DNA polymerase specificity. Biochemistry 45, 9675–9687.

49. Sucato, C. A., Upton, T. G., Kashemirov, B. A., Batra, V. K., Martínek, V., Xiang, Y., Beard, W. A., Pedersen, L. C., Wilson, S. H., McKenna, C. E., Florián, J., Warshel, A., and Goodman, M. F. (2007) Modifying the βγ leaving-group bridging oxygen alters nucleotide incorporation efficiency, fidelity, and the catalytic mechanism of DNA polymerase δ. Biochemistry 46, 461–471.

50. Santoso, Y., Joyce, C. M., Potapova, O., Le Reste, L., Hohlbein, J., Torella, J. P., Grindley, N. D., and Kapanidis, A. N. (2010) Conformational transitions in DNA polymerase I revealed by single-molecule FRET. Proc. Natl. Acad. Sci. U.S.A. 107, 715–720.

51. Bakhtina, M., Roettger, M. P., Kumar, S., and Tsai, M. D. (2007) A unified kinetic mechanism applicable to multiple DNA polymerases. Biochemistry 46, 5463–5472.

52. Bebenek, K., García-Díaz, M., Zhou, R.-Z., Povirk, L. F., and Kunkel, T. A. (2010) Loop 1 modulates the fidelity of DNA polymerase λ. Nucleic Acids Res. 38, 5419–5431.

53. Mejia, E., Burak, M., Alonso, A., Larraga, V., Kunkel, T. A., Bebenek, K., and García-Díaz, M. (2014) Structures of the Leishmania infantum polymerase β. DNA Repair 18, 1–9.

54. Osheroff, W. P., Beard, W. A., Wilson, S. H., and Kunkel, T. A. (1999) Base substitution specificity of DNA polymerase δ9252 depends on interactions in the DNA minor groove. J. Biol. Chem. 274, 20749–20752.

55. Osheroff, W. P., Beard, W. A., Yin, S., Wilson, S. H., and Kunkel, T. A. (2000) Minor groove interactions at the DNA polymerase δ active site modulate single-base deletion error rates. J. Biol. Chem. 275, 28033–28038.

56. Li, S. X., Vaccaro, J. A., and Sweasy, J. B. (1999) Involvement of phenylalanine 272 of DNA polymerase β in discriminating between correct and incorrect deoxynucleoside triphosphates. Biochemistry 38, 4800–4808.

57. Beard, W. A., Batra, V. K., and Wilson, S. H. (2010) DNA polymerase structure-based insight on the mutagenic properties of 8-oxoguanine. Mutat. Res. 703, 18–23.

58. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res. 35, W375–W383.

59. Bienstock, R. J., Beard, W. A., and Wilson, S. H. (2014) Phylogenetic analysis and evolutionary origins of DNA polymerase X-family members. DNA Repair 22, 77–88.