Kinetic Effect of a Downstream Strand and Its 5′-Terminal Moieties on Single Nucleotide Gap-filling Synthesis Catalyzed by Human DNA Polymerase λ*

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During short-patch base excision repair, the excision of a 5′-terminal 2-deoxyribose-5-phosphate moiety of the downstream strand by the 5′-2-deoxyribose-5-phosphate lyase activity of either DNA polymerase β or λ is believed to occur after each respective enzyme catalyzes gap-filling DNA synthesis. Yet the effects of this 5′-terminal 2-deoxyribose-5-phosphate moiety on the polymerase activities of these two enzymes have never been quantitatively determined. Moreover, x-ray crystal structures of truncated polymerase λ have revealed that the downstream strand and its 5′-phosphate group of gapped DNA interact intensely with the dRPase domain, but the kinetic effect of these interactions is unclear. Here, we utilized pre-steady state kinetic methods to systematically investigate the effect of a downstream strand and its 5′-moieties on the polymerase activity of the full-length human polymerase λ. The downstream strand and its 5′-phosphate were both found to increase nucleotide incorporation efficiency (k/p Kd) by 15- and 11-fold, respectively, with the increase procured by the effect on the nucleotide incorporation rate constant k/p rather than the ground state nucleotide binding affinity Kd. With 4 single nucleotide-gapped DNA substrates containing a 1,2-dideoxyribose-5-phosphate moiety, a 2-deoxyribose-5-phosphate, or apyrimidinic endonuclease, a DNA polymerase, a 5′-2-deoxyribose-5-phosphate lyase (dRPase), and a DNA ligase activity (21). Base excision repair (BER) is the major pathway to repair single base lesions (7). Short-patch and long-patch BER are the two subpathways of BER that remove and replace 1 (8–10) and 2–11 nucleotides (11–13), respectively. Short-patch BER starts with the excision of a modified base by a DNA glycosylase, leaving a noncoding apurinic or apyrimidinic site in DNA. This lesion is further processed and repaired by a 5′-acting apurinic or apyrimidinic endonuclease, a DNA polymerase, a 5′-2-deoxyribose-5-phosphate lyase (dRPase), and a DNA ligase (12, 14). It has been established that in mammalian systems DNA polymerase β (Fig. 1, Polβ), an X-family DNA polymerase, plays a critical role in short-patch BER (8, 9). The polymerase activity of Polβ catalyzes single nucleotide gap-filling synthesis (17), while its dRPase activity removes the 5′-terminal 2-deoxyribose-5-phosphate moiety (dRP) of a downstream strand (18). The uracil-initiated short-patch BER has been reconstituted in vitro by using purified recombinant human enzymes (19–21), and its reaction sequences (Scheme 1) have been established from steady state kinetic studies (21).

Like Polβ, the full-length DNA polymerase λ (fPolλ), a recently discovered member of the X-family DNA polymerases, also contains a dRPase (8 kDa) and a DNA polymerase (31 kDa) domain on its C terminus (Fig. 1) (22–25). In addition, fPolλ possesses a nuclear localization signal motif, a breast cancer susceptibility gene 1 C-terminal (BRCT) domain, and a proline-rich domain. Although the biological role of fPolλ has not been clearly identified, it is plausible that fPolλ contributes to BER because it shares 33% sequence identity to Polβ and possesses two key enzymatic activities required by BER. This hypothesis is directly supported by the following observations: (i) recombination human fPolλ purified from Escherichia coli can replace human Polβ in an in vitro reconstituted short-patch BER assay (26); (ii) mouse embryonic fibroblast Polβ−/− cell extract contains a substantial amount of active fPolλ that can also replace Polβ in a similar in vitro reconstituted BER assay, and monoclonal antibodies against fPolλ in this cell extract strongly reduce in vitro BER (27). fPolλ, like Polβ, lacks 3′ → 5′ exonuclease activity (22–24) and has low processivity when copying non-gapped or large gap DNA (25). With short gap DNA, the downstream strand, especially one with a 5′-phosphate, has...
been shown to increase polymerase processivity of both Polβ (28) and fPolα (25, 29). Moreover, Polβ has been found to incorporate a single nucleotide ~10-fold more efficiently with single nucleotide-gapped DNA than non-gapped DNA (30, 31), but the polymerase fidelity is not altered (31). Structural evidence suggests that the increase in polymerase processivity and efficiency is due to additional contacts that are established in a gapped DNA substrate between the dRPase domains of these two enzymes and the downstream strand (29, 32). The terminal 5′-phosphate on the downstream strand is buried in a positively charged pocket of the dRPase active site (29, 32, 33). So far, the effects of a downstream strand and its 5′-phosphate on the efficiency and fidelity of gap-filling synthesis catalyzed by fPolα has not been quantitatively determined. Here, these effects associated with human fPolα will be investigated through pre-steady state kinetic studies.

Moreover, steady state kinetic studies have identified the rate-limiting step in the reconstituted and uracil-initiated BER system (Scheme 1) as the removal of the dRP moiety by Polβ (21). fPolα is estimated to possess a 4-fold slower dRPase activity than Polβ (26), while these two enzymes catalyze single nucleotide gap-filling DNA synthesis with <2-fold difference in catalytic efficiency (34). These kinetic data strongly indicate that the 5′-dRP moiety in the downstream strand is removed after Polβ or fPolα fills single nucleotide-gapped DNA in Scheme 1. However, the effect of the 5′-dRP moiety on the fidelity and efficiency of either Polβ or fPolα has never been kinetically evaluated. In this report, we will use single nucleotide-gapped DNA substrates containing a dRP mimic on the 5′-terminus of the downstream strand to determine the kinetic effect of the 5′-dRP moiety on gap-filling DNA synthesis catalyzed by human fPolα through detailed pre-steady state kinetic analysis.

EXPERIMENTAL PROCEDURES

Materials—These chemicals were purchased from the following companies: [γ-32P]ATP, GE Healthcare; Biospin columns, Bio-Rad Laboratories; dNTPs, Invitrogen; T4 polynucleotide kinase, USB (Cleveland, OH). The full-length human Polα was cloned, expressed, and purified as described previously (34).

Synthetic Oligonucleotides—The DNA substrates shown in Fig. 2 were purchased from Integrated DNA Technologies (Coralville, IA) and purified by denaturing polyacrylamide gel electrophoresis (18% acrylamide, 8 M urea). Their concentrations were determined by measuring UV absorbance at 260 nm with calculated molar extinction coefficients. The primer strand 21-mer was 5′-32P-labeled by incubation with T4 polynucleotide kinase and [γ-32P]ATP for 1 h at 37 °C. The unreacted [γ-32P]ATP was subsequently removed by centrifugation through a Biospin-6 column (Bio-Rad). The 5′-32P-labeled primer 21-mer was then annealed with the corresponding non-radio-labeled downstream strand 19-mer to the template 41-mer at a molar ratio of 1.0:1.25:1.15, respectively, to form a 21–19/41-mer single nucleotide-gapped substrate (the top strand was composed of two oligonucleotides with a single nucleotide gap). Mixtures to be annealed were denatured at 95 °C for 8 min and then cooled slowly to room temperature over several hours.

Optimized Reaction Buffer L—All kinetic experiments were performed in buffer L containing 50 mM Tris-Cl (pH 8.4 at 37 °C), 5 mM MgCl2, 100
**RESULTS**

**Model DNA Substrates**—Based on the steady state kinetic results (21), the natural DNA substrate for the polymerase activity of Polα in BER is a dRP-DNA-type substrate (Fig. 4A) that exists as a mixture of different chemical species in solution (see “Discussion”) (35). To simplify kinetic analysis, we prepared four H-DNA substrates, shown in Fig. 2B, whose downstream strands contained a 5’-terminal 1,2-dideoxyribose-5-phosphate moiety that mimicked 5’-dRP. The H-DNA substrates are stable and exist in one chemical form in solution due to the lack of C1-hydroxyl group in their dRP-mimic moiety. To examine the kinetic effect of the downstream strand and its 5’-phosphate, we designed another gapped substrate, D-1OH (Fig. 2D). This substrate contained a downstream 19-mer that was not 5’-phosphorylated as in D-DNA (D-1) (Fig. 2C) and non-gapped D-1N (Fig. 2E), which lacked a downstream strand.

**Kinetic Effect of a dRP Mimic**—Previously, we have established a minimal kinetic mechanism (Scheme 2) for dTTP incorporation onto d-DNA (D-1) (Fig. 2C) catalyzed by the C-terminal Polβ-like domain of human Polα (Fig. 1, tPolα) (36). This scheme shows that an incoming dNTP binds to the tPolα-D-1 binary complex to establish a rapid equilibrium prior to nucleotide incorporation. We have also demonstrated that human fPolα follows the same minimal mechanism shown in Scheme 2 (34). This mechanism allows us to measure the apparent affinity of dNTP (Kd) for the Polα-DNA binary complex via the dNTP concentration dependence of the observed single turnover rate constant (kobs) (see “Experimental Procedures”).

Under single turnover conditions, a preincubated solution of 30 nM 5’-32P-labeled H-7 (Fig. 2B) and 150 nM human fPolα was reacted with increasing concentration of 32P-labeled dATP in buffer L (see “Experimental Procedures”). The single turnover method was employed because DNA dissociates from fPolα with a dissociation rate constant (k5) that is only ~2- to 3-fold slower than the maximum nucleotide incorporation rate constant (kobs), rendering the burst phase insignificant. Thus, the experiments were performed with fPolα in molar excess over DNA to allow the direct observation of nucleotide incorporation in a single pass of the reactants through the enzymatic pathway without complications resulting from the steady state formation of products (37). The DNA product 22-mer, rather than the template 41-mer, was 5’-32P-labeled.
Kinetic Studies of the Impact of a Downstream Strand

The short-patch base excision repair pathway involves at least five enzymes that catalyze six reaction steps, including two catalyzed by Polβ (Scheme 1). Many lines of biochemical and in vitro evidence (22–27, 38, 39) indicate that the polymerase and dRPase activities of Polα can be catalyzed by these two BER steps in vitro and may do so in vivo. Under the in vitro reconstituted BER reaction conditions, the velocity values for the uracil-DNA glycosylase, apurinic or apyrimidinic endonuclease, the DNA polymerase and dRPase activities of Polβ, and DNA ligase I have been measured to be 420, 100, 4.5, 0.75, 4.0 nmol/s, respectively (21). The rate-limiting step is identified as the dRP excision reaction that occurs at a similar velocity as the overall reconstituted BER system (0.6 nmol/s) (21). In addition, the polymerase activity of Polβ that catalyzes the single nucleotide gap-filling synthesis is 6-fold faster than its dRPase activ-

FIGURE 3. Concentration dependence on the pre-steady state rate constant of correct nucleotide incorporation. A, a preincubated solution of fPolα (150 nM) and 5'32P-labeled H-7 (30 nM) was rapidly mixed with increasing concentrations of Mg2+ (0.05 M, A 1 M, B 2 M, X 4 M, D 8 M, ○ 12 M, ○ 25 M, ● 48 M, □ 72 M) for various time intervals. The solid lines are the best fits to Equation 2, yielding a $k_p$ of 0.40 ± 0.01 s$^{-1}$ and a $K_d$ of 0.78 ± 0.08 M. The kinetic parameters (Table 1) for the incorporation of each of the remaining three correct nucleotides (dTTP onto H-1, dCTP onto H-6, and dGTP onto H-8) and for the incorporation of each of the 12 possible misincorporations onto H-1, H-6, H-7, and H-8 (Fig. 2B) were carried out in the same manner as described above. Notably, the ground state binding affinity of all nucleotides to fPolα(2H-DNA) (Table 1) was within 10-fold, but the $k_p$ values of correct nucleotides were three to five orders of magnitude higher than those of incorrect nucleotides. The differences in $k_p$ and $K_d$ led to significantly different nucleotide incorporation efficiencies ($k_p/K_d$) between correct and incorrect nucleotide incorporations and resulted in a fidelity of gap-filling synthesis in the range of $10^{-4}$ to $10^{-5}$ (Table 1).

Kinetic Effect of the 5'-Phosphate of a Downstream Strand—The kinetic parameters of nucleotide incorporation onto D-1OH (Fig. 2D) were measured with fPolα under the single turnover conditions described above and listed in Table 2. The apparent binding affinity of all four nucleotides was unaffected by the absence of the 5'-phosphate of the 19-mer in D-1OH, but the $k_p$ values were lowered by 10- to 100-fold (Table 2). Thus, the substrate specificity ($k_p/K_d$) of matched and mismatched nucleotides with D-1OH was 10- and 100-fold smaller, respectively, than those with D-DNA (D-1) (Table 2). Notably, dATP incorporation onto D-1OH was too slow to be measured. A slightly larger effect on misincorporations compared with correct dTTP incorporation resulted in a slightly higher fidelity with D-1OH than with D-DNA (D-1).

Kinetic Effect of a Downstream Strand—The significant kinetic effect of the 5'-phosphate moiety suggested the downstream strand itself could have a dramatic influence on the single nucleotide gap-filling efficiency of fPolα. To examine this hypothesis, we measured the pre-steady state kinetic parameters (Table 2) of single nucleotide incorporation onto D-1OH that lacked a downstream strand (Fig. 1E) by employing single turnover experiments as described above. The correct dTTP was incorporated at a rate constant of 0.025 s$^{-1}$ and a ground state binding affinity of 2.6 M (Table 2). In comparison with the kinetic data with D-1OH (Table 2), the lack of a downstream strand further decreased the incorporation efficiency of matched dATP and mismatched dCTP by approximately an additional 10-fold each. Incorporations of mismatched dGTP and dATP onto D-1OH were too slow to be observed in several hours.

DISCUSSION

The short-patch base excision repair pathway involves at least five enzymes that catalyze six reaction steps, including two catalyzed by Polβ (Scheme 1). Many lines of biochemical and in vitro evidence (22–27, 38, 39) indicate that the polymerase and dRPase activities of Polα are able to catalyze these two BER steps in vitro and may do so in vivo. Under the in vitro reconstituted BER reaction conditions, the velocity values for the uracil-DNA glycosylase, apurinic or apyrimidinic endonuclease, the DNA polymerase and dRPase activities of Polβ, and DNA ligase I have been measured to be 420, 100, 4.5, 0.75, 4.0 nmol/s, respectively (21). The rate-limiting step is identified as the dRP excision reaction that occurs at a similar velocity as the overall reconstituted BER system (0.6 nmol/s) (21). In addition, the polymerase activity of Polβ that catalyzes the single nucleotide gap-filling synthesis is 6-fold faster than its dRPase activi-
ity which cleaves the dRP moiety on the 5’ terminus of the downstream strand. This suggests that the natural single nucleotide-gapped DNA for the polymerase activity of Polβ should contain a dRP moiety on its downstream strand. Thus, the best model DNA substrates are the dRP-DNA substrates (Fig. 2A) rather than the D-DNA substrates (Fig. 2C). However, because the 2-deoxyribose moiety in dRP-DNA is an equilibrium mixture of α- and β-hemiacetals (2-deoxy-β-erythro-pentofuranoses), an aldehyde, and a hydrated aldehyde in solution (35), rigorous kinetic analysis becomes overly complicated; as such, D-DNA substrates have been predominantly used as the gapped DNA substrates to examine the kinetics of nucleotide incorporation catalyzed by both Polβ (30, 31, 40–44) and Polλ (34, 36). To overcome the instability of dRP-DNA in solution, we decided to use the H-DNA substrates (Fig. 2B) in our pre-steady state kinetic analysis with human fPolλ.

Under single turnover conditions, the kinetic parameters of all 16 possible nucleotide incorporations were measured using the H-DNA substrates (Fig. 2B), and the fidelity of human fPolλ was determined to be in the range of $10^{-4}$ to $10^{-5}$ (Table 1). This range was identical to the fidelity with D-DNA (34), suggesting the presence of the dRP mimic did not affect the gap-filling error frequency of fPolλ. However, the substrate specificity values (Table 1) are 1.6- to 6.7-fold lower than those corresponding values for nucleotide incorporation onto D-DNA (34), resulting in an average efficiency ratio, $(k_p/K_d)_{D-DNA}/(k_p/K_d)_{H-DNA}$, of 3.4 (Table 1). The difference in substrate specificity $(k_p/K_d)$ can be mathematically attributed to either $k_p$ or $K_d$ or both. However, the ground state binding affinities $(K_d)$ of both correct and incorrect nucleotides with H-DNA (Table 1) are very similar to those corresponding $K_d$ values obtained with D-DNA (34), indicating that the presence of the dRP mimic on the 5’ terminus of the downstream strand did not affect the binding affinity of an incoming nucleotide. In contrast, the 16 maximum incorporation rate constants $(k_p)$ with H-DNA on average were 3.5-fold lower than those corresponding $k_p$ values.

### Table 1

| dNTP      | $K_d$ (µM) | $k_p$ (s⁻¹) | $(k_p/K_d)_{H-DNA}$ | Fidelity | $(k_p/K_d)_{D-DNA}$ | Efficiency ratio |
|-----------|------------|-------------|---------------------|----------|---------------------|-----------------|
| **Template A (H-1)** |
| dATP      | 1.7 ± 0.3  | 0.5 ± 0.3   | 0.30                | 1        | 1.5                 | 5.1             |
| dCTP      | 10 ± 5     | 2.7 ± 1.0   | 5 × 10⁻⁴           | 8.7 ± 10⁻⁵ | 5.0 ± 10⁻⁵ | 2.0             |
| dGTP      | 6.4 ± 0.6  | 2.5 × 10⁻⁴ | 1 × 10⁻⁴           | 1.3 × 10⁻⁴ | 1.3 × 10⁻⁴ | 3.4             |
| **Template G (H-6)** |
| dCTP      | 1.6 ± 0.3  | 1.1 ± 0.04  | 0.69                | 1        | 1.8                 | 2.6             |
| dATP      | 1.6 ± 0.6  | 1.0 × 10⁻⁵ | 9 × 10⁻⁷           | 9.5 × 10⁻⁶ | 3.3 × 10⁻⁵ | 5.0             |
| dCTP      | 10 ± 3     | 1.4 ± 1.0   | 2 × 10⁻⁴           | 1.9 × 10⁻⁴ | 2.4 × 10⁻⁴ | 2.1             |
| dGTP      | 4.2 ± 0.7  | 4.7 × 10⁻⁴ | 3 × 10⁻⁴           | 1.6 × 10⁻⁵ | 5.0 × 10⁻⁵ | 4.5             |
| **Template T (H-7)** |
| dATP      | 0.78 ± 0.08| 0.40 ± 0.01 | 0.49                | 1        | 1.6                 | 3.3             |
| dCTP      | 11 ± 2     | 7.1 × 10⁻⁵ | 6 × 10⁻⁶           | 6.4 × 10⁻⁶ | 1.3 × 10⁻⁵ | 2.4 × 10⁻⁵ | 3.7             |
| dGTP      | 11 ± 2     | 4.8 × 10⁻⁵ | 4 × 10⁻⁴           | 4.5 × 10⁻⁴ | 9.3 × 10⁻⁴ | 7.0 × 10⁻⁴ | 1.6             |
| dCTP      | 8 ± 5      | 2.2 × 10⁻³ | 4 × 10⁻⁴           | 2.7 × 10⁻⁴ | 5.6 × 10⁻⁴ | 1.4 × 10⁻³ | 5.1             |
| **Template C (H-8)** |
| dGTP      | 1.5 ± 0.3  | 0.76 ± 0.02 | 0.51                | 1        | 1.2                 | 2.3             |
| dATP      | 1.5 ± 0.3  | 3.8 × 10⁻⁵ | 2 × 10⁻⁵           | 2.5 × 10⁻⁵ | 4.9 × 10⁻⁵ | 1.7 × 10⁻⁴ | 6.7             |
| dCTP      | 3 ± 1      | 6.2 × 10⁻⁴ | 4 × 10⁻⁵           | 1.9 × 10⁻⁴ | 3.7 × 10⁻⁴ | 3.8 × 10⁻⁴ | 2.0             |
| dGTP      | 9 ± 5      | 6 × 10⁻⁴   | 2 × 10⁻⁴           | 7.3 × 10⁻⁵ | 1.4 × 10⁻⁴ | 1.9             |

* Calculated as $(k_p/K_d)_{H-DNA}/[(k_p/K_d)_{correct} + (k_p/K_d)_{incorrect}]$.
* The $(k_p/K_d)_{D-DNA}$ values are from Table I of Ref. 34.
* Calculated as $(k_p/K_d)_{H-DNA}/(k_p/K_d)_{H-DNA}$. **Table 2**

| dNTP      | $K_d$ (µM) | $k_p$ (µM⁻¹ s⁻¹) | $(k_p/K_d)_{H-DNA}$ | Fidelity |
|-----------|------------|------------------|---------------------|----------|
| **D-DNA (D-1) substrate** |
| dATP      | 2.6 ± 0.4  | 3.9 ± 0.2        | 1.5                 | 1        |
| dCTP      | 14.8 ± 5.0 | 0.022 ± 0.003    | 1.5 × 10⁻³          | 9.8 × 10⁻⁴ | 5.8 × 10⁻⁴ | 5.4 × 10⁻⁴ |
| dGTP      | 8.2 ± 0.6  | 0.006 ± 0.003    | 7.6 × 10⁻⁴          | 1.1 × 10⁻⁴ | 1.4 × 10⁻⁴ | 1.9             |
| dATP      | 4.1 ± 0.5  | 0.0029 ± 0.0001  | 7.1 × 10⁻⁴          | 1.1 × 10⁻⁴ | 1.4 × 10⁻⁴ | 1.9             |
| **D-1OH substrate** |
| dATP      | 4.1 ± 0.8  | 0.56 ± 0.03      | 0.14                | 1        |
| dCTP      | 5.5 ± 1.1  | (1.6 ± 0.1) × 10⁻⁴ | 2.9 × 10⁻⁵          | 2.1 × 10⁻⁴ | 5.8 × 10⁻⁴ | 5.4 × 10⁻⁴ |
| dGTP      | 8.5 ± 2.7  | (1.3 ± 0.1) × 10⁻⁴ | 1.5 × 10⁻⁶          | 1.1 × 10⁻⁴ | 1.4 × 10⁻⁴ | 1.9             |
| **D-1N substrate** |
| dATP      | 2.6 ± 0.7  | 0.025 ± 0.002    | 9.4 × 10⁻³          | 1        |
| dCTP      | 6.6 ± 2.0  | (8.3 ± 0.6) × 10⁻⁴ | 1.3 × 10⁻⁶          | 1.4 × 10⁻⁴ | 1.9             |

* Calculated as $(k_p/K_d)_{H-DNA}/[(k_p/K_d)_{correct} + (k_p/K_d)_{incorrect}]$.
* Data are from Table I of Ref. 34.
with D-DNA, suggesting the presence of the dRP mimic modestly affected catalysis during nucleotide incorporation. However, what is the catalytic efficiency of fPol with the natural dRP-DNA substrates (Fig. 2A)? Because of the close chemical similarity between dRP-DNA and H-DNA (Fig. 2), it is reasonable to speculate that fPol will incorporate nucleotides onto dRP-DNA with efficiencies closer to those observed with H-DNA than with D-DNA. This hypothesis goes against the qualitative experiments of Srivastava et al. (21) in which they have observed similar nucleotide incorporation efficiency with Polβ in the presence or absence of the dRP moiety. It is known that the dRP excision catalyzed by the dRPase domain occurs via a Schiff-base formation and β-elimination (45–47). If the Schiff-base formation between the C1 of the dRP group and Lys-312 of fPol (29, 48) is faster than nucleotide incorporation while the β-elimination limits both dRP excision and BER, it is possible that fPol will be more efficient with dRP-DNA than with H-DNA because the covalent anchoring of the downstream strand of dRP-DNA may facilitate gap-filling DNA synthesis catalyzed by fPol. More kinetic experiments are required to examine the aforementioned hypothesis. From a structural perspective, the effect of the dRP moiety or its mimic on DNA, dNTP binding, and the polymerase active site conformation remains unclear, because all ternary crystal structures of both tPol (33, 49) and Polβ (32, 50, 51) are solved in the presence of “D-DNA,” rather than “dRP-DNA” or “H-DNA.” However, these structures do reveal intimate contacts between the dRPase domains of both Polβ and tPol (Fig. 1) and the downstream strand (29, 32). The terminal 5′-phosphate of the downstream strand is buried in a positively charged pocket of the dRPase active site (29, 32), e.g. Tyr-267, Arg-275, Tyr-279, Lys-307, and Arg-308 in tPol (29, 48). Thus, the dRP mimic likely interacts with the dRPase domain, which in turn may affect the active site conformation of fPol and thus the $k_p$ value of nucleotide incorporation.

Consistently, the intimate interactions between the dRPase domain, the downstream strand, and its 5′-phosphate moiety did impact catalysis significantly. Table 3 indicates that fPol incorporated a matched dTTP most efficiently with single nucleotide-gapped D-DNA (D-1) (Fig. 2C). The absence of the 5′-phosphate group in D-1OH (Fig. 2D) caused an 11-fold decrease in dTTP incorporation efficiency ($k_p / K_o$), whereas lack of the entire downstream strand in D-1N (Fig. 2E) led to an additional 15-fold decrease. As a consequence, the catalytic efficiency decreased by 160-fold from D-DNA (D-1) to D-1N. Similarly, Polβ is found to have 6- to 40-fold higher nucleotide incorporation efficiency with single nucleotide-gapped DNA than with non-gapped DNA (52, 53). The low substrate specificity of matched dTTP with D-1N further suggested that fPol is too inefficient to be a primer/template-dependent polymerase. In contrast, the dTTP incorporation efficiency of fPol (1.5 μM$^{-1}$s$^{-1}$) is close to the range of Polβ (1.9–8.5 μM$^{-1}$s$^{-1}$) in the presence of single nucleotide-gapped DNA (12, 14–16). Moreover, our recent fidelity studies suggest that human fPol has similar single nucleotide gap-filling fidelity (10$^{-4}–10^{-5}$) as Polβ (34). Taken together, these results strongly suggested that fPol, like Polβ, preferred short gapped DNA over non-gapped DNA and was likely a gap-filling polymerase involved in BER.

Interestingly, from D-1N to D-1OH and to D-DNA (D-1), the change in dTTP incorporation efficiency was due to a 100-fold variation in $k_p$, while the binding of an incoming nucleotide ($K_o$) was similarly tight (Table 2). In contrast, the increase of catalytic efficiency from non-gapped to gapped DNA with Polβ was due to a considerable change in $K_d$ rather than $k_p$ (12, 14–16). These suggested that fPol and Polβ achieve higher catalytic efficiency through different mechanisms and these two enzymes have evolved divergently. Similar $K_d$ values of dTTP with three different DNA substrates (Table 3) indicated that the unprecedentedly high nucleotide binding affinity was due to the intimate interactions between an incoming nucleotide and the active site residues of fPol as revealed by the ternary crystal structures of fPol-DNA (33), rather than the presence of a downstream strand and its 5′'-phosphate moiety. The significant decrease in $k_p$ from D-DNA (D-1) to D-1OH and to D-1N (Table 3) suggested the intense interactions between the downstream strand, including its 5′'-phosphate and the dRPase domain, as demonstrated by the binary and ternary crystal structures of fPol (29, 33), directed and anchored the productive binding of DNA and dNTP at the active site of fPol. Consequently, the absence of these interactions will either improperly align the 5′'-OH of the upstream primer 21-mer and the α-phosphate of the dNTP for in-line attack or affect the local protein conformational change, leading to slower catalysis. In the meantime, however, the fidelity of fPol (Table 2) was slightly increased. This trend is consistent with what we have observed with different truncated fragments of Polα (34) but disagrees with the general trend summarized from a survey of the A-, B-, X-, and Y-families by Beard et al. (54) that a more catalytically efficient DNA polymerase has a higher polymerization fidelity.

In conclusion, our pre-steady state kinetic data have demonstrated that the downstream strand and its 5′'-phosphate moiety are critical to the polymerase efficiency of fPol. For the first time, we have quantitatively evaluated the kinetic effect of a dRP mimic on the 5′ terminus of a downstream strand. Because this dRP mimic only modestly affected the incorporation efficiency of both correct and incorrect nucleotides while having insignificant effect on the fidelity of human DNA polymerase λ, gapped substrates like D-DNA in Fig. 2C, which have been predominantly used in the literature, are reasonable model substrates.

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