Contribution of the Reverse Rate of the Conformational Step to Polymerase β Fidelity[†]

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ABSTRACT: A complete understanding of the kinetic mechanism of fidelity requires comparison of correct and incorrect dNTP incorporation pathways in both the forward and reverse directions. The studies presented here focus on the dNTP-induced conformational step, which has historically been proposed by many to be the major determinant of fidelity. As it was recently highlighted [Tsai, Y. C., and Johnson, K. A. (2006) *Biochemistry 45*, 9675−9687], chemistry can be the slowest step in the forward direction of the correct dNTP incorporation pathway, yet the corresponding microscopic rate constant would not contribute toward fidelity in the case when the reverse rate of the conformational step is slower than chemistry. Here we use a stopped-flow technique to directly measure the reverse rate of the conformational step in the DNA polymerase β (Pol β) kinetic pathway. Extensive pre-steady-state kinetic studies presented include the utilization of 2-aminopurine-labeled DNA substrates, 2-aminopurine nucleotide triphosphate, a nonhydrolyzable nucleotide analogue dAMPCPP, and a rapid sequential mixing reaction scheme. Additionally, the effect of mismatched dNTPs, various metal ions, and the presence of the 3′-terminal hydroxyl group of the primer on the rate of the reverse "opening" conformational step were analyzed. Our analyses indicate that reverse "opening" is drastically facilitated in the presence of mismatched ternary complexes, which is in agreement with the hypothesis that the ternary complex is destabilized by the presence of incorrect dNTP. By analysis of the relative magnitudes of chemistry and reverse "opening" in the presence of both matched and mismatched matched ternary complexes, this work further validates that, for Pol β, fidelity is dictated by the differences in free energy required to reach the highest energy transition state of the chemical step.

DNA polymerases, enzymes that play a central role in DNA replication and repair, are required to possess sufficient accuracy to maintain genome invariability. Long remaining an issue of great importance, the kinetic mechanism of high-fidelity DNA replication has been the subject of numerous studies (for review see refs *1* and *2*). A complete understanding of the kinetic mechanism of DNA polymerase fidelity requires determination of the free energy profiles (or microscopic rate constants for each step) for correct and incorrect nucleotide incorporation reactions (*2, 3*). On the basis of earlier studies of Pol I (Klenow fragment) and T7 DNA polymerase, it has been proposed that DNA polymerases utilize an induced-fit mechanism of fidelity that involves a rate-limiting conformational step (*4–7*). Parallel structural studies of several polymerases have strengthened the induced-fit hypothesis by demonstrating that correct nucleotide binding induces a large conformational rearrangement of the polymerase subdomains, which brings the enzyme to an active "closed" conformation (*8, 9*). However, later studies have indicated that this subdomain closing conformational change is a relatively fast step for a number of DNA polymerases (*10–13*). Extensive studies of mammalian DNA polymerase β (Pol β)[1] in our laboratory have shown that the chemical step is rate-limiting for both matched and mismatched dNTP incorporation, and therefore the chemical transition state plays the central role in mismatched dNTP discrimination (*2, 14*). Our conclusions have found additional support from the findings of other research groups (*15–18*). However, it has been recently emphasized (*13, 19*) that despite the fact that the chemical step of the correct nucleotide incorporation is slower than the forward rate of the conformational step ("closing"), if the reverse rate of the conformational step ("opening") is slower than chemistry, the microscopic rate constant of the chemistry step will not be present in the $k_{pol}/K_{d\ app}$ expression for correct nucleotide insertion and, therefore, in the fidelity expression $(k_{pol}/K_{d\ app})^{cor}/(k_{pol}/K_{d\ app})^{inc}$. This justifies the necessity of further investigation into the contribution of the reverse "opening" step to Pol β fidelity.

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[1] Abbrevations: 2-AP, 2-aminopurine; dNTP, 2′-deoxynucleoside 5′-triphosphate; d(2-AP)TP, 2-aminopurine 2′-deoxyribose 5′-triphosphate; dAMPCPP, 2′-deoxyadenosine 5′-[(α,β)-methylene]triphosphate; Pol β, *Rattus norvegicus* DNA polymerase β.

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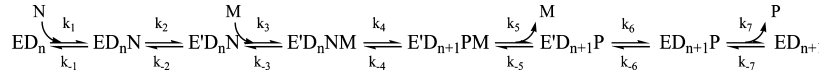
Scheme 1: Kinetic Scheme of Single dNTP Incorporation Catalyzed by Pol β[a]



[a] E = Pol β in open conformation; E′ = closed conformation; D_n = DNA; N = Mg·dNTP; M = catalytic Mg^{2+}; P = Mg·PP_i.

Table 1: Sequences of DNA Substrates[a]

19dd/36AP(A)	5′-GCCTCGCAGCCGTCCAAC**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTGG**A**Ã**A**TCAGTGGAGTTAGGT
19dd/36AP(C)	5′-GCCTCGCAGCCGTCCAAC**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTGG**C**Ã**A**TCAGTGGAGTTAGGT
19dd/36AP(G)	5′-GCCTCGCAGCCGTCCAAC**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTGG**G**Ã**A**TCAGTGGAGTTAGGT
19dd/36AP(T)	5′-GCCTCGCAGCCGTCCAAC**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTGG**T**Ã**A**TCAGTGGAGTTAGGT
19/36AP(T)	5′-GCCTCGCAGCCGTCCAACC 3′-CGGAGCGTCGGCAGGTTGG**T**Ã**A**TCAGTGGAGTTAGGT
19dd/36(T)	5′-GCCTCGCAGCCGTCCAAC**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTGG**T**ATCAGTGGAGTTAGGT
18dd/36(G)	5′-GCCTCGCAGCCGTCCAA**C**$_{dd}$ 3′-CGGAGCGTCGGCAGGTTG**G**TATCAGTGGAGTTAGGT

[a] The first number in the title designation represents the number of bases in the primer; the second number represents the number of bases in the template. Substrates with dd designation have a dideoxy-terminated primer terminus. The letter in parentheses indicates the templating base. In the DNA sequences, Ã refers to the 2-aminopurine base, and C_{dd} represents 2′,3′-dideoxycytidine 5′-monophosphate.

As shown in our earlier studies of Pol β, single dNTP incorporation by Pol β results in a biphasic fluorescence change as revealed by fluorescence stopped-flow assay (*20, 21*). These studies indicate that the fast fluorescence phase corresponds to the nucleotide-induced conformational step to form the closed ternary complex prior to chemistry and that the slow fluorescence transition corresponds to subdomain reopening after the rate-limiting chemical step (*10, 22*). Our current knowledge can be summarized as the kinetic mechanism of single dNTP incorporation presented in Scheme 1. Nucleotide binding to the Pol β·DNA binary complex (step 1) induces a fast conformational change (step 2), which makes possible the binding of the catalytic metal ion (step 3) and brings the enzyme into an active conformation poised for the rate-limiting chemical step (step 4). Following catalytic metal dissociation (step 5), a relatively fast "reopening" step occurs (step 6), and PP_i product release (step 7) concludes the cycle of single turnover. In a recent publication, we provided evidence that Pol β catalyzed mismatched and matched dNTP incorporations proceed via analogous kinetic pathways (*14*). Importantly, it was demonstrated that the rate of the conformational change induced by mismatched dNTP is comparable to that of subdomain closing induced by correct dNTP. The kinetic analyses further suggested that in the presence of mismatched dNTP the closed ternary complex and the transition state for the chemical step are much less stable compared to those with matched dNTP.

The mechanism of DNA polymerase catalysis involves two metal ions (*9, 23, 24*), both of which likely contribute to the associative transition state stabilization. One metal ion is involved in binding with the triphosphate moiety of dNTP, and the other plays an essential role in coordinating the 3′-OH nucleophile and α-phosphate for phosphoryl transfer. In our earlier studies of Pol β we demonstrated that while the second (catalytic) metal ion is essential for catalysis, the nucleotide-induced conformational "closing" step requires only the nucleotide binding metal ion (*22*). As shown for several polymerases, productive nucleotide substrate binding is possible only in the form of a metal−dNTP complex (*22, 25, 26*). Therefore, removal of all divalent metal ions from the reaction mixture should abolish dNTP-induced conformational "closing". Here, using this knowledge, we designed stopped-flow experiments to directly measure the reverse rate of the dNTP-induced conformational step. Comparison of the rates of the reverse conformational "opening" and chemistry under a variety of the reaction conditions shows that the rate of reverse "opening" is faster than or comparable to the rate of chemistry, which suggests that chemistry remains a key determinant of Pol β fidelity.

MATERIALS AND METHODS

Materials. The nonhydrolyzable nucleotide analogue dAMP-CPP was obtained from Jena Bioscience GmbH; d(2-AP)TP was from TriLink BioTechnologies; illustra solution dNTPs were from GE Healthcare; reverse-phase C_{18} cartridges were from Waters. Materials and reagents not listed here were of standard molecular biology grade.

Enzymes. The R258A and I260Q mutants of Pol β were obtained by site-directed mutagenesis as described (*10, 14*). The recombinant wild-type rat Pol β and the mutants were overexpressed and purified as previously described from the BL21(DE3)pLysS *Escherichia coli* strain (*27*). The enzyme concentrations were determined by UV absorbance at 280 nm using an extinction coefficient of 21200 M^{-1} cm^{-1}. The enzymes appeared to be >95% homogeneous on the basis of SDS−PAGE developed using the silver staining method.

DNA Substrates. The sequences of primer/template DNA substrates used in this study are shown in Table 1. Custom synthesized oligomers were purchased from Integrated DNA Technologies. Each DNA oligomer was further purified by denaturing PAGE (15−18% acrylamide, 7 M urea) and desalted using a Sep-Pak C_{18} cartridge. Oligomer concentrations were determined by UV absorbance at 260 nm with the extinction coefficients provided by the manufacturer. Primer and template were annealed in a 1.1:1 molar ratio, respectively.

Stopped-Flow Fluorescence Assays. Experiments were performed on an Applied Photophysics SX 18MV stopped-flow apparatus. The excitation wavelengths were 285 and 312 nm for tryptophan and 2-aminopurine, respectively, with a spectral bandpass of 4 nm. Tryptophan emission was monitored using a 335 nm high pass filter (Corion), and 2-aminopurine emission was monitored using a 360 nm high pass filter (Corion). Typically, profiles were collected on a 1s logarithmic time scale, and a minimum of seven runs were performed and averaged.

The standard assay buffer consisted of 50 mM Tris-HCl, 20 mM BisTris-HCl, 1 mM DTT, and 10% (w/v) glycerol, with the ionic strength adjusted to 81 mM with KCl, and pH adjusted to 7.6 (at 37 °C). The buffer was filtered with a 0.45 μm filter and degassed prior to use. Reverse reaction preparations utilized a stock solution of 0.500 M EDTA made from EDTA dipotassium salt and adjusted to a pH of 7.5 with KOH. All reactions were performed at 37 °C.

A typical forward reaction was initiated by rapid mixing of two solutions in the assay buffer described above: (a) 400 nM DNA substrate and 1 μM DNA polymerase and (b) suitable dNTP or nucleotide analogue. In all forward reactions, both solutions possess equal $MgCl_2$ concentrations. A typical reverse reaction assay involved rapid mixing of two solutions in the standard assay buffer described above: (a) 400 nM DNA substrate, 1 μM DNA polymerase, suitable dNTP or nucleotide analogue, and 5 mM $MgCl_2$ (or as specified otherwise) and (b) 20 mM EDTA.

Sequential Mixing Stopped-Flow Assays. Experiments were performed using an SQ.1 sequential mixing accessory. Specifically, Pol β•19/36AP(T) binary complex was pre-mixed with dATP in the presence of 7 mM $MgCl_2$; then, after 15−500 ms of delay, the reverse reaction was initiated by addition of 20 mM EDTA, and 2-AP fluorescence was monitored. The DNA substrate utilized in these assays retained the hydroxyl group at the 3′-terminus of the primer.

Data Analysis. Kinetic traces were fit using Applied Photophysics software to a single or a double exponential equation:

$$\text{fluorescence} = A_1 e^{-k_1 t} + C \quad (1)$$

$$\text{fluorescence} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C \quad (2)$$

where k_1 and k_2 are the rate constants, A_1 and A_2 are the corresponding amplitudes, and C is an offset constant.

RESULTS

Removal of Divalent Metal Ions Induces Reverse "Opening" of the Ternary Complex. The rate of the reverse conformational "opening" step (step 2 in Scheme 1) can be measured by removal of the nucleotide substrate from the reaction mixture containing the preformed E′•D_n•N ternary complex. Conformational closing of the fingers subdomain requires binding of the nucleotide substrate in complex with a divalent metal ion (*22, 25, 26*). In the absence of divalent metal ions, the enzyme likely does not bind dNTP productively, and the conformational change does not occur. Thus addition of EDTA to the preformed Pol β ternary complex should shift the equilibrium to the open state and thus allow measurement of the rate of the reverse "opening" step. This

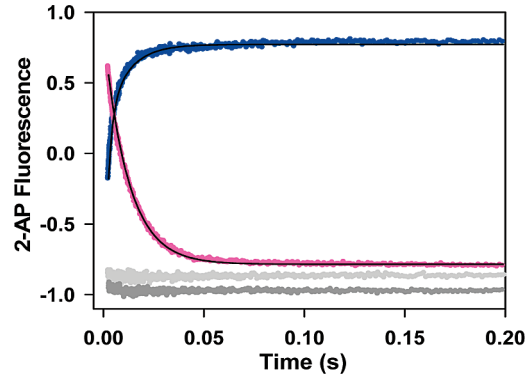


FIGURE 1: Forward "closing" and reverse "opening" in the presence of correct dNTP as monitored by stopped-flow 2-AP fluorescence assay. The forward reaction (dark blue) was initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(T), and 5.4 mM $MgCl_2$) with solution B (700 μM dATP and 5.4 mM $MgCl_2$). Double exponential fit (black line) yielded rates of 490 ± 22 s^{-1} and 95.9 ± 3.0 s^{-1}. The corresponding reverse reaction (pink) was initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(T), 700 μM dATP, and 5.7 mM Mg^{2+}) with solution B (20 mM EDTA). Single exponential fit (black line) yielded a rate of 82.6 ± 0.3 s^{-1} for the reverse reaction. Gray traces represent controls of the reverse reaction where either dNTP (light gray) or Mg^{2+} (dark gray) was omitted from the reaction mixture.

was experimentally verified: When the preformed ternary complex (Pol β, dideoxy-terminated 19dd/36AP(T) DNA, and correct dATP in the presence of Mg^{2+}) was mixed with EDTA, a 2-AP fluorescence change with a single exponential rate of 82.6 ± 0.3 s^{-1} was observed (Figure 1A, pink trace). The direction of the fluorescence change was opposite to that elicited by the forward reaction initiated by mixing the preformed Pol β•19dd/36AP(T) binary complex with dATP in the presence of Mg^{2+} (Figure 1A, dark blue trace). This observation is consistent with the proposal that the observed fluorescence decay depicted by the pink trace in Figure 1 actually represents the reverse of Mg•dNTP-induced conformational closing. We performed additional control reactions where either dATP or Mg^{2+} was omitted from the reaction mixture. In both controls, addition of EDTA did not induce any significant fluorescence change. Therefore, the observed decay of 2-AP fluorescence is due to depletion of Mg•dATP substrate from the reaction mixture.

Reverse "Opening" Is Facilitated for a Mismatched Ternary Complex. Reverse "opening" experiments starting from other matched and mismatched ternary complexes were also performed. The results for all 16 base pairs are shown in Figure 2. For the T•G, T•C and T•T mismatched complexes, addition of EDTA results in a fluorescence decay with a single exponential rate of ~500 s^{-1}, which is more than 5 times faster than the rate of the fluorescence change observed with the T•A matched ternary complex under identical conditions (Figure 2A). The significantly faster rate constants obtained for the mismatched reverse conformational step likely result from the destabilization of the mismatched ternary complex (*14*). The results in the other three panels (B−D) are similar to that in (A), except no detectable fluorescence change was observed for the C•C base pair. The rates obtained from Figure 2 are listed in Table 2.

Interestingly enough, different matched base pairs demonstrated differences in the rate of the reverse "opening". In agreement with the fact that C•G and G•C base pairs are more thermodynamically stable compared to T•A and A•T,

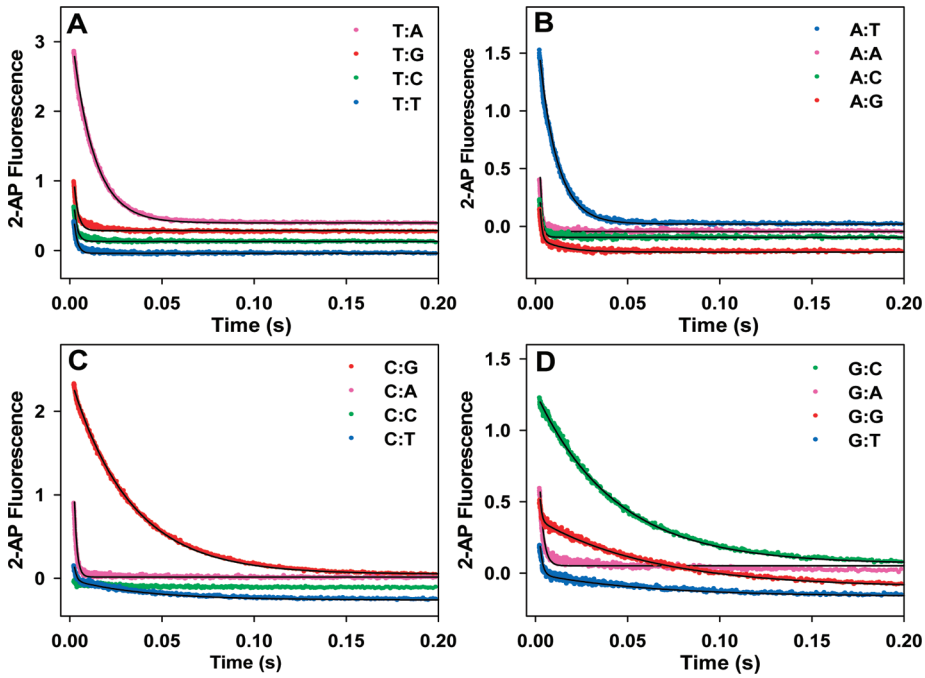


FIGURE 2: Effect of different matches/mismatches on reverse "opening". The reverse reaction was initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(X), 5 mM free Mg^{2+}, and either 700 μM match or 4 mM mismatch dNTP) with solution B (20 mM EDTA). Black lines show single or double exponential fit and the obtained rate constants are listed in Table 2.

Table 2: Reverse "Opening" Rate Constants (s^{-1}) for 16 Base Pairs[a]

	dNTP substrate			
DNA substrate	dATP	dTTP	dGTP	dCTP
19dd/36AP(T)	84.3 ± 0.3	565 ± 12	455 ± 11	574 ± 12
19dd/36AP(A)	900 ± 15	103 ± 0.5	998 ± 40 81 ± 5	696 ± 16
19dd/36AP(C)	700 ± 7	782 ± 33 25 ± 1	31.2 ± 0.1	n/d[b]
19dd/36AP(G)	474 ± 9	662 ± 22 16 ± 1	1010 ± 50 16 ± 0.2	23.3 ± 0.1

[a] Values for the reverse "opening" were obtained from single or double exponential fit (eqs 1 and 2) of the stopped-flow data presented in Figure 2. [b] Not determined due to a small change in 2-AP fluorescence.

the rate of the EDTA induced "opening" for C•G and G•C was 31.2 s^{-1} and 23.3 s^{-1} compared to 84.3 s^{-1} and 103 s^{-1} for T•A and A•T, correspondingly (Table 2). Under the same condition, the rate of nucleotide incorporation was 13.8 s^{-1} and 17.6 s^{-1} for T•A and A•T and 8.9 s^{-1} and 3.7 s^{-1} for C•G and G•C base pairs, correspondingly (each rate was determined in a stopped-flow assay of single nucleotide incorporation (not shown) as the rate of the slow fluorescence transition).

Dependence of Reverse Conformational Step on Altered Reaction Parameters. In order to provide further evidence that the fluorescence decay observed upon EDTA addition is representative of the reverse conformational step, the reverse reaction was investigated under multiple reaction conditions including varied concentrations of EDTA, dNTP, enzyme, and DNA. Based upon the kinetic scheme depicted in Scheme 2, it is expected that the rate of the reverse "opening" reaction should not depend on EDTA, dNTP, DNA, or enzyme concentration.

First, the reverse reaction was examined as a function of increasing concentrations of the correct dATP (Figure 3A). A control reaction with 0 mM dATP did not demonstrate

Scheme 2: Simplified Model for the Reverse "Opening" Reaction[a]

$$E'\ DNA_{dd}N \rightleftharpoons E\ DNA_{dd}N \xrightarrow[Mg^{2+}]{} E\ DNA_{dd} + dNTP$$

[a] DNA_{dd} = dideoxy-terminated DNA.

any fluorescence change (Figure 3A, gray trace). The rate of reverse "opening" did not show significant change over a range of 10−500 μM dATP (Table 3). At the same time, the amplitude of the fluorescence change did show a dependence on the nucleotide concentration. The observed increase in amplitude is expected since as the concentration of dATP increases, the concentration of ternary complex formed prior to mixing with EDTA increases as well. The same results were obtained in the analysis of mismatched dGTP concentration dependence−independence of the rate on dGTP concentration and an increase in fluorescence change amplitude with increasing dGTP concentration (Figure 3B).

Also, the effect of different EDTA concentrations on the reverse conformational step was examined. The rate of the reverse conformational step for the matched nucleotide did not show significant change over a range of 15−70 mM EDTA (Figure 3C; only representative data are shown). In addition, the observed rate of the fluorescence change was not altered by changes in DNA or enzyme concentration (data not shown).

Use of Tryptophan Fluorescence. We also utilized fluorescence from Pol β's single Trp^{325} residue to examine the effect of EDTA addition on the preformed Pol β•DNA_{dd}•MgdNTP ternary complex. While 2-AP fluorescence senses changes in DNA structure, Trp^{325}, located on the fingers subdomain of Pol β, acts as a parallel reporter for conformational changes occurring in the protein portion of the ternary complex (*20*). Figure 4A presents stopped-flow traces

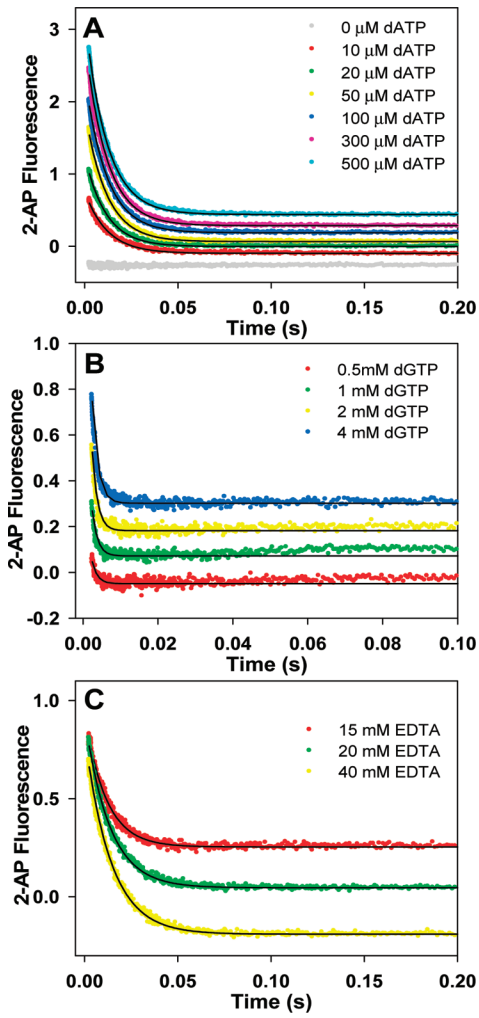


FIGURE 3: Effect of altered reaction parameters on reverse "opening". (A) The matched dNTP concentration dependence reverse reactions were initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(T), 5 mM free Mg^{2+}, and dATP varied) with solution B (40 mM EDTA). (B) The mismatched dNTP concentration dependence reverse reactions were initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(T), 5 mM free Mg^{2+}, and dGTP varied) with solution B (20 mM EDTA). (C) The EDTA concentration dependence reverse reactions in the presence of correct dNTP were initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19dd/36AP(T), 600 μM dATP, and 10 mM Mg^{2+}) with solution B (containing varied EDTA). Black lines represent fit to a single exponential equation (eq 1), and the obtained amplitudes and rate constants are listed in Table 3.

for the forward (dark blue trace) and reverse (pink trace) reactions as monitored by Trp fluorescence using an 18dd/36(G) DNA substrate, which provides the best signal-to-noise ratio for Trp fluorescence. Similar to 2-AP fluorescence, in response to EDTA addition, Trp fluorescence changed in the direction opposite to the fluorescence change of the forward reaction (Pol β·18dd/36(G)·MgdCTP ternary complex formation). To verify that both fluorophores reported the rate of the same step, we compared rates determined from both reporters, 2-AP and Trp, in stopped-flow fluorescence assays using similar DNA substrates. To accomplish this, we utilized 19dd/36(T) nonlabeled DNA substrate, which has the same sequence as the DNA used in 2-AP fluorescence experiments except that 2-AP at the +1 position is substituted with adenine. The obtained rate of 75.6 s^{-1} for the matched dATP was in excellent agreement with the rate of the reverse "opening" step (82.6 s^{-1}) obtained in stopped-flow 2-AP fluorescence assays. Therefore, both fluorescent probes likely reflect the same step, involving both local and global changes in the Pol β·DNA_{dd}·MgdNTP ternary complex.

The Reverse "Opening" Step Involves Spatial Movements in the Ternary Complex. Since reverse conformational "opening" should involve a large conformational motion, the rate of this motion is expected to be sensitive to altered buffer viscosity, similar to that observed for the conformational "closing" during the forward reaction of dNTP incorporation (*22*). Evaluation of the viscosity effect on the reverse reaction was conducted by applying variable concentrations of glycerol in the reaction buffer (Figure 4B). The rate of reverse reaction for the matched nucleotide substrate demonstrated a marked dependence on glycerol concentration: as the concentration of glycerol increased, the rate of the fluorescence change decreased. The same trend was observed for mismatched dNTP (data not shown).

R258A Mutation Facilitates the Reverse Conformational "Opening" Step. Results of our previous studies suggest that the R258A mutant exhibits facilitated reopening after nucleotide incorporation (*10*). It is expected that this mutation would have a similar effect on the reverse rate of the conformational step before chemistry. Upon examination, we found that, for all correct base pairs, the fluorescence change induced by addition of EDTA to the preformed R258A·DNA_{dd}·MgdNTP complex demonstrated an increase in rate in comparison to the corresponding reaction with WT Pol β. As a representative example, the green trace in Figure 5 shows the stopped-flow trace for the T·A base pair.

I260Q Mutation Reduces the Rate of the Reverse Conformational "Opening" Step. In our recent publications, we demonstrated that I260Q has a nonperturbed forward rate of conformational "closing" and suggested that I260Q mutant may have a stabilized closed conformation due to formation of a hydrogen-bonding network between the side chains of Gln^{260}, Tyr^{296}, Glu^{295}, and Arg^{258} (*14, 28*). In support of this interpretation, the reverse conformational step for the matched dNTP was slowed for this mutant compared to WT Pol β (purple trace in Figure 5).

Effect of Different Divalent Metal Ions on the Rate of the Reverse Conformational "Opening" Step. As demonstrated for several polymerases, application of Mn^{2+} instead of Mg^{2+} can stabilize the closed ternary complex (*29–31*). In agreement with this, the reverse reaction in the presence of 1 mM Mn^{2+} gave the rate constant equal to 39.1 s^{-1}, which is 2-fold slower than the reaction in the presence of Mg^{2+} (Figure 6A).

It is known that Ca^{2+} does not support Pol β catalyzed dNTP incorporation. However, structural studies have demonstrated that Pol β·DNA can properly bind Ca·dNTP to form a ternary complex (*32*). Here we first examined the forward reaction and found that rapid mixing of the Pol β·19/36AP(T) binary complex with matched dATP in the presence of Ca^{2+} resulted in the fast phase of the fluorescence change only (black trace in Figure 6B). Significantly, the DNA substrate used in this assay retained the 3′-OH group, yet the slow phase of the fluorescence change was not observed. This finding is in agreement with the proposal that Ca^{2+} does not support chemistry but supports nucleotide binding and the following conformational "closing" step.

Table 3: Reverse "Opening" Rate Constants and Amplitudes of 2-AP Fluorescence Change Obtained with Various Correct and Incorrect Mg·dNTP and EDTA Concentrations[a]

dATP (correct)	10 μM	20 μM	50 μM	100 μM	300 μM	500 μM
rate (s^{-1})	83.6 ± 0.8	88.4 ± 0.5	87.6 ± 0.5	86.7 ± 0.4	86.9 ± 0.4	85.9 ± 0.4
amplitude	0.86 ± 0.004	1.25 ± 0.004	1.83 ± 0.005	2.18 ± 0.006	2.59 ± 0.006	2.75 ± 0.006
dGTP (incorrect)	0.5 mM	1 mM	2 mM	4 mM		
rate (s^{-1})	705 ± 57	690 ± 25	700 ± 18	654 ± 11		
amplitude	0.56 ± 0.1	1.1 ± 0.09	2.0 ± 0.1	2.31 ± 0.08		
EDTA	15 mM	20 mM	40 mM			
rate (s^{-1})	88.2 ± 0.9	76.0 ± 0.5	72.0 ± 0.4			

[a] Single exponential fit (eq 1) of the stopped-flow data presented in Figure 3. 19/36AP(T) DNA substrate was used. EDTA concentration dependence was studied in the presence of 600 μM dATP.

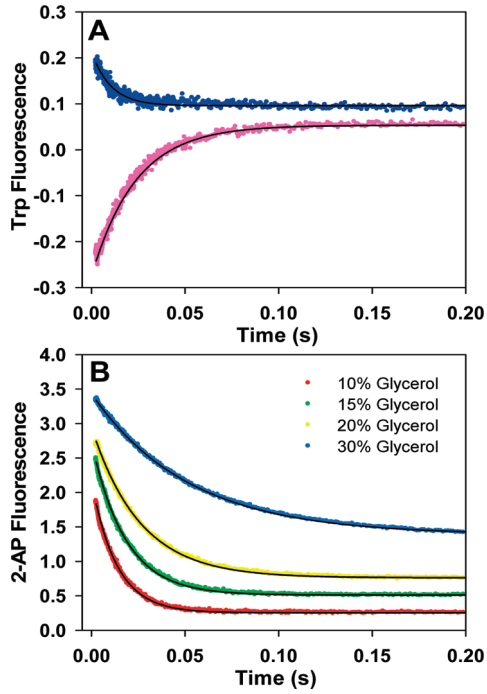


FIGURE 4: (A) Forward "closing" and reverse "opening" for correct dNTP as monitored in stopped-flow tryptophan fluorescence assay. The forward reaction (dark blue) was initiated by rapid mixing of solution A (2 μM Pol β, 1.8 nM 18dd/36(G), and 5.4 mM $MgCl_2$) with solution B (700 μM dCTP and 5.4 mM $MgCl_2$). The reverse reaction (pink) was initiated by rapid mixing of solution A (2 μM Pol β, 1.8 nM 18dd/36(G), 700 μM dCTP, and 5.7 mM Mg^{2+}) with solution B (20 mM EDTA). Single exponential fit (black lines) yielded a rate of 42.5 ± 0.4 s^{-1} for the reverse reaction and a rate of 97.5 ± 2.6 s^{-1} for the forward reaction. (B) Viscosity dependence of reverse "opening" of the matched ternary complex Pol β·19dd/36AP(T)·MgdATP. Solution A (1 μM Pol β, 400 nM 19dd/36AP(T), 400 μM dATP, and 10 mM Mg^{2+}) was rapidly mixed with solution B (20 mM EDTA), and 2-AP fluorescence was monitored. Both solutions A and B contained equal indicated concentrations of glycerol in the Pol β assay buffer. Single exponential fit (black lines) yielded rates of 74.7 ± 0.3 s^{-1}, 55.6 ± 0.2 s^{-1}, 38.7 ± 0.1 s^{-1}, and 19.3 ± 0.1 s^{-1} for 10%, 15%, 20%, and 30% glycerol, respectively.

In the reverse "opening" reaction, when a preformed Pol β·19/36AP(T)·CadATP ternary complex was rapidly mixed with EDTA, a fluorescence change with a faster rate of 570 s^{-1} was observed (cyan trace in Figure 6B). This lies in agreement with the proposal that the bulkier Ca^{2+} perturbs the active site by destabilizing the closed ternary complex (*32*). Attempting to probe the role of the primer's 3′-OH group in closed ternary complex stability, we compared the reverse rate obtained for 19/36AP(T) and 19dd/36AP(T)

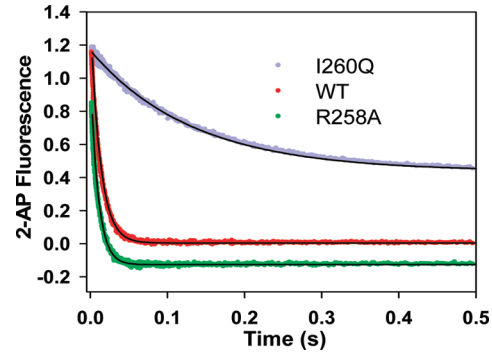


FIGURE 5: Comparison of reverse "opening" conformational step for I260Q (purple), R258A (green), and WT Pol β (red) in the presence of correct dNTP. The reverse reaction was initiated by rapid mixing of solution A (1 μM enzyme, 400 nM 19dd/36AP(T), 700 μM dATP, and 7 mM $MgCl_2$) with solution B (20 mM EDTA). Single exponential fit (black lines) yielded rates of 79.2 ± 0.3 s^{-1}, 102 ± 0.5 s^{-1}, and 7.56 ± 0.05 s^{-1} for WT, R258A, and I260Q, respectively.

DNA in the presence of Ca^{2+}. The use of the dideoxy-terminated DNA resulted in very similar rate (592 s^{-1}) of fluorescence change induced by EDTA. This might indicate that Ca^{2+} binds the Pol β active site in a different manner than Mg^{2+}, such that it is not involved in interactions with the 3′-OH group. This assumption is consistent with results from structural studies of Pol β which report that only the nucleotide binding site is occupied by a metal ion in the presence of Ca^{2+} (*32*).

Application of dNTP Analogues in the Reverse Conformation Step Studies. 2-Aminopurine is an excellent adenine analogue and widely used as a fluorescent label of DNA. However, the use of deoxy-2-aminopurine triphosphate (d(2-AP)TP) in fluorescence studies of DNA polymerase is very limited (*26, 33, 34*). Binding of Mg·d(2-AP)TP to the Pol β·19dd/36(T) binary complex resulted in decrease of fluorescence intensity (Figure 7A). The observed quench of fluorescence intensity is likely caused by stacking interactions of d(2-AP)TP with DNA substrate and enzyme active site residues, similar to the interactions observed in crystal structures of the Pol β closed ternary complex indicating that subdomain closure induced by nucleotide binding "sandwiches" the incoming dNTP between the 3′-terminal primer base and Asp^{276} (*9, 35*). Note that conventional reaction conditions when dNTP is in excess over Pol β·DNA did not provide satisfactory results (not shown). However, the use of excess of Pol β·DNA over d(2-AP)TP provided excellent signal-to-noise ratios.

Addition of EDTA to the preformed Pol β·19dd/36(T)·Mgd(2-AP)TP ternary complex resulted in an increase

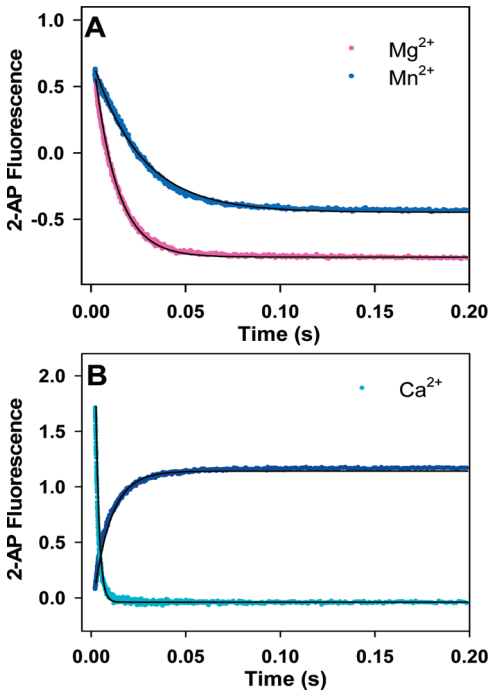


FIGURE 6: Effect of Mn^{2+} (panel A) and Ca^{2+} (panel B) on the rate of the reverse "opening" for correct dNTP. Reactions were initiated by rapid mixing of solution A (1 μM Pol β, 400 nM DNA, and 700 μM dATP in the presence of corresponding metal ion) with solution B (40 mM EDTA). Dideoxy-terminated 19dd/36AP(T) DNA was used for reactions in the presence of either Mg^{2+} or Mn^{2+}, while 19/36AP(T) was utilized in Ca^{2+} experiments. In (B) the dark blue and cyan traces correspond to the forward and reverse reactions. Black lines represent fit to a single exponential equation (eq 1), and the obtained rate constants were 109 $\pm$ 0.8 s^{-1} for the forward reaction in the presence of Ca^{2+} and 82.6 $\pm$ 0.3 s^{-1}, 39.1 $\pm$ 0.2 s^{-1}, and 570 $\pm$ 3 s^{-1} for the of the reverse "opening" in the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+}, respectively.

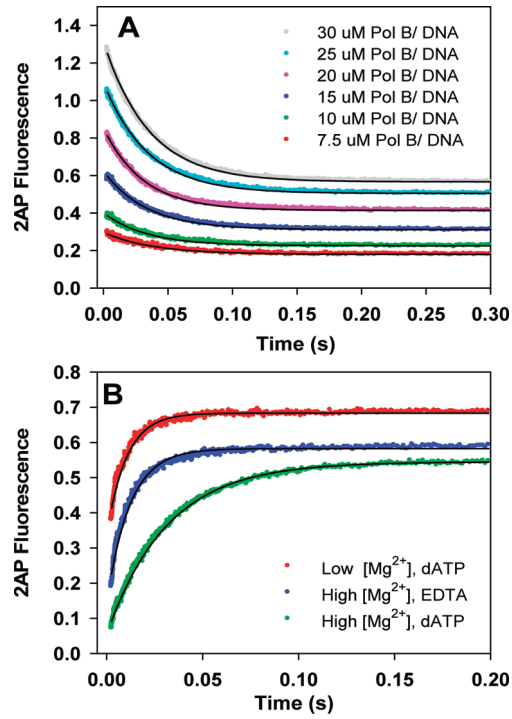


FIGURE 7: Application of d(2-AP)TP nucleotide analogue. (A) d(2-AP)TP binding to the Pol β·19dd/36(T) binary complex. The reaction was initiated by rapid mixing of solution A (varied concentration of 1:1 complex of Pol β and 19dd/36(T), 10 mM Mg^{2+}) with solution B (containing 2 μM d(2-AP)TP and 10 mM Mg^{2+}). Single exponential fit (black lines) yielded rates of 26.1 $\pm$ 0.3 s^{-1}, 30.0 $\pm$ 0.3 s^{-1}, 29.8 $\pm$ 0.2 s^{-1}, 32.6 $\pm$ 0.2 s^{-1}, 29.1 $\pm$ 0.1 s^{-1}, and 28.7 $\pm$ 0.1 s^{-1}, for 7.5, 10, 15, 20, 25, and 30 μM Pol β·DNA, respectively. (B) Reverse reaction initiated by mixing of solution A (30 μM Pol β, 25 μM 19dd/36(T), 2 μM d(2AP)TP, and 10 mM $MgCl_2$) with solution B, containing either 20 mM EDTA (red) or 1 mM dATP and 10 mM $MgCl_2$ (blue). The green trace represents the reverse reaction initiated by rapid mixing of solution A (30 μM Pol β, 25 μM 19dd/36(T), 2 μM d(2AP)TP, and 0.2 mM $MgCl_2$) with solution B (1 mM dATP, 1 mM $MgCl_2$). Single exponential fit (black lines) yielded rates of 83.5 $\pm$ 0.8 s^{-1}, 31.9 $\pm$ 0.1 s^{-1}, and 97.7 $\pm$ 1.1 s^{-1} for addition of EDTA, dATP and 10 mM $MgCl_2$, and dATP and 1 mM $MgCl_2$, respectively.

of 2-aminopurine fluorescence intensity (Figure 7B, red trace). Similarly, when competitive correct nucleotide (dATP) was used to displace d(2-AP)TP from the ternary complex, an increase in fluorescence intensity was observed (Figure 7B, green trace). However, the rate of the fluorescence change was nearly 3-fold slower than the rate of d(2-AP)TP release induced by EDTA under identical conditions (10 mM Mg^{2+}). Importantly, under conditions of low free Mg^{2+} concentration, the d(2-AP)TP dissociation rate was comparable for both dATP and EDTA (Figure 7B, blue trace). This observation could be explained by the obligatory dissociation of the catalytic Mg^{2+} from the Pol β active site prior to the reverse conformational "opening". Catalytic Mg^{2+} dissociation is presumably a very fast process, and addition of EDTA instantly prevents rebinding of the catalytic metal ion. On the other hand, in the case of the d(2-AP)TP release upon dATP addition, excess Mg^{2+} remains in the reaction mixture. This potentially shifts the equilibrium toward the closed ternary complex with two Mg^{2+} bound and slows the reverse conformational "opening" step.

Use of Nonhydrolyzable dNTP Analogues. The use of nonhydrolyzable dNTP analogues provides an opportunity to study the reverse conformational step starting from the ternary Pol β·DNA·MgdNTP complex with nonterminated primer. dAMPCPP is a nonhydrolyzable analogue of dATP in which the α,β bridging oxygen is substituted with a CH_2 group. Similar types of analogues have been recently used to obtain high-resolution crystal structures of the Pol β ternary complex with both nucleotide binding and catalytic metal ions present in the active site (*29, 36*). To our knowledge, nonhydrolyzable analogues have not yet been applied in stopped-flow fluorescence analyses of DNA polymerases.

Figure 8A shows stopped-flow traces for the forward reaction of the Pol β·19/36AP(T) binary complex with dATP (black trace) and dAMPCPP (red trace). Our earlier studies of the Pol β kinetic mechanism have suggested that the fast phase of the biphasic fluorescence change, observed during the course of matched dATP incorporation (black trace), corresponds to the prechemistry conformational step (step 2 in Scheme 1), whereas the slow phase of the fluorescence originates from the conformational reopening step after chemistry (step 6 in Scheme 1). The rate of the slow phase of fluorescence matches the rate of chemistry by virtue of the fact that the subdomain reopening is normally limited by slower chemistry. In agreement with the nonhydrolyzable nature of dAMPCPP, Pol β is not able to catalyze the nucleotidyl transfer step (chemistry), and therefore, the slow phase is not present in the red trace (Figure 8A).

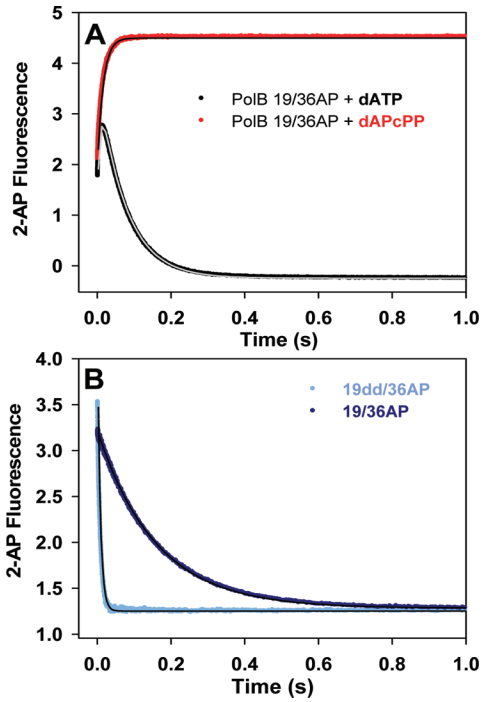


FIGURE 8: Application of dAMPCPP nucleotide analogue. (A) Reaction of dATP (black) and dAMPCPP (red) with Pol β·19/36AP(T) binary complex. The reaction was initiated by rapid mixing of solution A (1 μM Pol β, 400 nM 19/36AP(T), and 5 mM $MgCl_2$) with solution B (containing 700 μM dATP or dAMPCPP and 5 mM Mg^{2+}). Double exponential fit (white line) of the dATP incorporation trace yielded rates of 140 ± 1 s^{-1} and 13.7 ± 0.1 s^{-1} for the fast and slow phases, correspondingly. Single exponential fit (black line) of the forward reaction with dAMPCPP yielded rate of 64.1 ± 0.3 s^{-1}. (B) Reverse reaction initiated by addition of 20 mM EDTA to preformed matched Pol β·DNA·MgdAMPCPP complex (1 μM Pol β, 400 nM DNA, 700 μM dAMPCPP, and 5 mM $MgCl_2$) with either 19/36AP(T) (dark blue) or 19dd/36AP(T) (light blue) DNA substrate. Single exponential fit (black lines) yielded rates of 6.32 ± 0.02 s^{-1} and 131 ± 1 s^{-1} for 19/36AP(T) and 19dd/36AP(T) DNA, respectively.

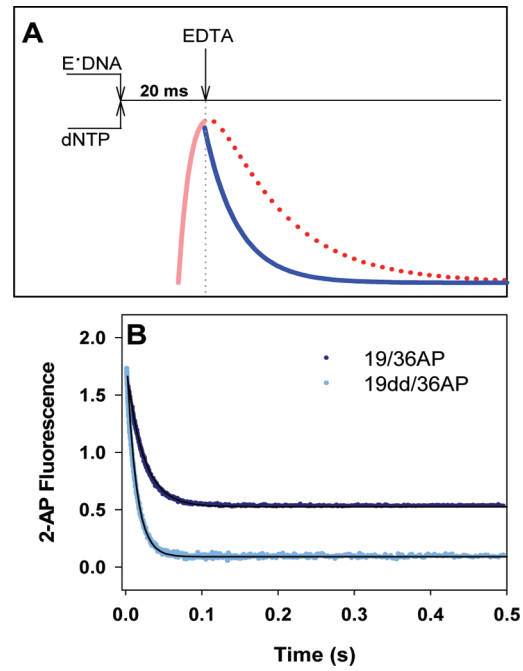


FIGURE 9: Sequential mixing stopped-flow assay. (A) Experimental design. The red solid curve corresponds to the reaction during delay time, which is not detected in the sequential stopped-flow experiment. The blue curve corresponds to the reverse reaction initiated by EDTA addition (detectible). The red dotted curve corresponds to the forward reaction of dNTP incorporation, which occurs if EDTA is omitted from solution C. (B) Results of sequential mixing assay with non-dideoxy-terminated 19/36AP(T) DNA (dark blue) and dideoxy-terminated 19dd/36AP(T) DNA (light blue). Solution A, containing 2 μM Pol β, 800 nM DNA, and 7 mM $MgCl_2$, was first rapidly mixed with solution B, containing 1.4 mM dATP (correct nucleotide) and 7 mM $MgCl_2$. After a delay of 18 ± 2 ms, the reverse reaction was initiated by rapid mixing with solution C, containing 20 mM EDTA. Single exponential fit (black lines) yielded rates of 44.7 ± 0.1 s^{-1} and 81.3 ± 0.3 s^{-1} for 19/36AP(T) and 19dd/36AP(T) DNA, respectively.

The reverse reaction, initiated by EDTA addition to the preformed Pol β·19/36AP(T)·MgdAMPCPP ternary complex, produces a decrease in 2AP fluorescence intensity with a rate of 6.32 s^{-1} (Figure 8B, dark blue trace). This is an order of magnitude slower than the rate of the fluorescence change in the similar reaction with dideoxy-terminated 19dd/36AP(T) DNA (Figure 8B, light blue trace), which suggests that the 3′-OH group contributes significantly toward stabilization of the closed ternary complex.

Application of a Sequential Mixing Stopped-Flow Assay To Examine the Contribution of the 3′-OH Primer Terminus. Previously, we utilized the sequential mixing mode of the stopped-flow instrument to study the Pol β reaction pathway in the forward direction (*22*). Here we employ this approach to initiate the reverse "opening" of the transiently formed closed ternary complex during the course of dNTP incorporation into a non-dideoxy-terminated DNA substrate. Figure 9A depicts the experimental design of the sequential mixing stopped-flow assay. Specifically, the Pol β·DNA binary complex was premixed with correct dNTP in the presence of Mg^{2+}; then, after 18 ± 2 ms of delay, the reverse reaction was initiated by rapid mixing with EDTA. It is reasonable to assume that EDTA addition instantly removes all free Mg^{2+} from the reaction mixture, which prevents catalysis and forces the reaction in the reverse direction. The delay time of 18 ± 2 ms was chosen because this time corresponds to the maximum 2-AP fluorescence in the forward reaction of dATP incorporation into 19/36AP(T) DNA, thus representing the time at which maximum accumulation of the transient closed conformation of the ternary complex is achieved.

As predicted from the experimental design, addition of EDTA to the briefly premixed Pol β·19/36AP and dATP results in a fluorescence decay with a rate of 44.7 s^{-1} (dark blue trace in Figure 9B). As the delay time increased, the amplitude of the fluorescence change decreased to the extent that after 500 ms delay no fluorescence change was observed. This observation is consistent with the fact that as the reaction progresses in the forward direction (upon premixing Pol β·19/36AP with dATP), the concentration of the transient closed ternary complex initially increases, reaching the maximum at 18 ± 2 ms, and then decreases to zero with the completion of the single nucleotide incorporation reaction. Importantly, the rate of the observed fluorescence change remained virtually identical at all delays examined (data not shown). The rate of the reverse "opening" obtained in the sequential mixing assays was 3.5-fold faster than the rate of dNTP incorporation under these conditions (k_{slow} = 12.8 s^{-1}). However, the use of non-dideoxy-terminated DNA resulted in a reverse "opening" rate approximately 2-fold slower than the rate of the same reaction using dideoxy-terminated

DNA substrate (light blue trace in Figure 9B). This agrees with the proposed stabilization role of the primer's 3′-OH group and is consistent with the results obtained in the aforementioned experiments utilizing nonhydrolyzable dNTP analogues.

DISCUSSION

Direct Measurement of the Reverse Rate of the Conformational Step. Experimental data presented in this paper provide supporting evidence that the fluorescence change observed in stopped-flow assays upon addition of EDTA to the preformed E′DN complex actually corresponds to the conformational change in the reverse direction (step 2, Scheme 1). First, the direction of fluorescence decay for reverse conformational "opening" is opposite that observed for forward conformational "closing" initiated by Mg•dNTP binding. Second, the step responsible for the observed fluorescence decay likely involves large physical movements because the rate of the fluorescence change was sensitive to reaction buffer viscosity (*22, 37*). Furthermore, the same rate was observed using both 2-aminopurine and tryptophan fluorescence, confirming that this step involves both local and global conformational rearrangements in the Pol β•DNA_{dd}•MgdNTP ternary complex. Third, as expected for the reverse step, the rate of the reaction initiated by addition of EDTA is independent of dNTP, DNA, and enzyme concentration.

Our observation of a faster rate of fluorescence decay in the reactions with mispaired incoming nucleotides correlates well with the generally accepted premise of the intrinsic instability of mismatched ternary complexes. In our recent publication, we reported similar forward rates for Pol β's conformational "closing" for both matched and mismatched dNTP incorporation (*14*), which suggests similar TS energy for the conformational step preceding chemistry. At the same time, a significant difference in the apparent dissociation constant ($K_{d\ app}^{dNTP}$) suggests a difference in the ground state energy of the matched and mismatched closed ternary complexes of Pol β (*14, 38*). Accordingly, the energy barrier for the reverse conformational "opening" is expected to be smaller and the corresponding rate faster for the reaction with a mispaired dNTP. This was confirmed for reverse reactions starting from different mismatched ternary complexes of Pol β. Our findings disagree with the conclusion from the recent analysis of T7 DNA polymerase using a single molecule technique, which implies that the forward rate of the conformational change is slowed down for mismatched dNTP, whereas the reverse "opening" rate does not change significantly for mismatched dNTP (*25*). Even though it is possible that the two enzymes possess distinct mechanisms for mismatch discrimination, another recent study of T7 DNA polymerase using a stopped-flow fluorescence technique demonstrated that the reverse rate of subdomain opening is substantially faster in the case of mismatched dNTP (*13*).

The method was used to enhance our understanding of the kinetic mechanism of R258A and I260Q, two well-characterized mutants of Pol β (*10, 14, 39–41*). MD simulation studies of Pol β suggested that R258 reorientation in the course of the subdomain closing conformational change could be the rate-limiting step in the correct dNTP incorporation pathway (*42*). However, recent kinetic analysis of the R258A variant suggested that reorientation of the Arg^{258} side chain is not rate-limiting in the overall kinetic pathway of Pol β, yet is kinetically significant in subdomain reopening after chemistry (*10*). Based upon this finding, it is reasonable to assume that the R258A mutant of Pol β would also possess facilitated conformational steps in the reverse direction. In this study, we found that the reaction initiated by addition of EDTA to the preformed R258A•DNA_{dd}•MgdNTP ternary complex was approximately 30% faster than the reaction with WT Pol β. This result further supports our previous conclusion that conformational steps are facilitated in the R258A mutant (*10*).

Resent SAXS and molecular modeling studies proposed that the glutamine substitution of the Ile260 might result in a more stable closed conformation of the mutant enzyme (*28*). Our kinetic analysis of the I260Q mutant also suggested lower energy of the closed ternary complex (*14*). The observation that I260Q demonstrated slower reverse "opening" is fully consistent with the conclusions of the aforementioned studies.

Effect of the 3′-OH Group on the Reverse Conformational "Opening". Absence of the primer 3′-OH group, which is intimately involved in the nucleotidyl transfer reaction, might result in distorted active site geometry (*36*) and potentially affect the stability of the closed ternary conformation. A striking example of the critical role of the 3′-OH has been demonstrated in studies of a low fidelity Dbh DNA polymerase from *Sulfolobus acidocaldarius* (*43*). In the absence of the 3′-OH, while the enzyme is still able to bind nucleotide substrate, it does not undergo the structural rearrangements required for the active enzyme conformation. On the other hand, results obtained with Pol β and Klenow fragment of *E. coli* Pol I suggested that the conformational "closing" remains largely unaffected regardless of the presence or absence of the 3′-OH primer terminus (*12, 22, 44, 45*). Results of the sequential mixing stopped-flow experiments presented here indicate that the presence of 3′-OH group is able to moderately stabilize the closed conformation of Pol β. Taken together, it appears that the 3′-OH group plays both catalytic and structural roles, a property of structure−function integration that has also been observed previously for enzymatic residues (*46*). Surprisingly, no difference between dideoxy-terminated and non-dideoxy-terminated DNA was observed in stopped-flow experiments where Mg^{2+} was substituted by Ca^{2+}. This may imply that Ca^{2+} does not coordinate the 3′-OH, which is consistent with the suggestion of earlier structural studies that Pol β's active site cannot accommodate two bulkier metal ions, thus explaining why only the nucleotide binding Ca^{2+} is present (*32*). Besides a larger ionic radius, Ca^{2+} demonstrates different coordination properties having a coordination number that varies from 6 to 8 depending on the specific protein environment (*47*). In any case, the significantly facilitated reverse "opening" in the presence of Ca^{2+} suggests a substantially perturbed conformation of the closed ternary complex.

The presence or absence of the 3′-OH group on the DNA primer could significantly affect the catalytic Mg^{2+} binding step. These studies did not include a detailed investigation of the influence of catalytic Mg^{2+} on the rate of the reverse "opening". It is reasonable that catalytic Mg^{2+} binding could shift the equilibrium toward the closed conformation, as was observed in stopped-flow experiments with the d(2AP)TP

Scheme 3: Simplified Kinetic Model for Single dNTP Incorporation[a]

$$E{\bullet}DNA_n + dNTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}DNA_n{\bullet}dNTP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F{\bullet}DNA_n{\bullet}dNTP \overset{k_3}{\rightleftharpoons} F\ {\bullet}DNA_{n+1} + PPi$$

[a] Scheme is reproduced from ref *13*.

analogue. However, it remains to be determined whether an alternative hypothesis can explain the effect of excess Mg^{2+} more adequately. It is possible that a ternary complex with two metal ions adopts a more stable conformation than a complex with only one metal ion, therefore undergoing reverse "opening" slower than the lesser complexed counterpart. Further experiments using methodology applied in these studies (application of nonhydrolyzable dNTP analogues, sequential-mixing technique) and possibly novel approaches are required to evaluate the kinetic role of the catalytic metal ion in the mechanism of Pol β.

Contribution of Chemistry and of the Reverse Conformational "Opening" to Pol β Fidelity. Fundamentally, DNA polymerase fidelity results from the free energy difference between the highest energy transition state in the reaction pathways for correct and incorrect nucleotide incorporation (*3*). However, discrimination against an incorrect substrate might be realized at all elementary steps of the reaction pathway. Moreover, the degree of discrimination at each step is likely different for different DNA polymerases (*2*). A complete understanding of the kinetic mechanism of fidelity requires determination of the free energy profiles (or rate constants for each step) for correct and incorrect nucleotide incorporation reactions.

DNA polymerase fidelity is defined as a ratio of catalytic efficiencies for correct versus incorrect dNTP incorporation. For a simplified mechanism shown in Scheme 3, polymerase catalytic efficiency can be expressed as

$$\frac{k_{pol}}{K_{d\ app}} = \frac{k_1 k_2 k_3}{k_2 k_3 + k_{-1}(k_{-2} + k_3)}$$

Clearly, the contribution of each rate constant depends on the relative magnitudes of the rate constants. For instance, as it was recently stressed by K. A. Johnson and co-workers (*13, 19*), if chemistry is much faster than the reverse rate of the conformational step ($k_3 \gg k_{-2}$), the specificity constant can be reduced to

$$\frac{k_{pol}}{K_{d\ app}} = \frac{k_1 k_2}{k_2 + k_{-1}}$$

in which case it is evident that the chemistry step (k_3) has no bearing on specificity. T7 DNA polymerase serves as a good example of this instance. As shown by Tsai et al. (*13*) in the reaction with correct dNTP, T7 demonstrates a much slower reverse "opening" rate compared to the rate of chemistry, whereas incorrect dNTP incorporation is characterized by slow chemistry and facilitated reverse "opening". Therefore, the authors concluded that T7 DNA polymerase fidelity is dictated by the rate of conformational "closing" for matched incorporation and by the conformational step equilibrium and the rate of chemistry for mismatched incorporation. It is important to point out that in this case the highest energy transition state of the correct dNTP incorporation pathway corresponds to the conformational step and the highest energy transition state of the incorrect dNTP incorporation pathway corresponds to chemistry. In contrast to T7 results, Pol β studies presented here demonstrate that the rate of reverse "opening" for matched nucleotides remains 3−5 times faster than chemistry. Still it is important to keep in mind that the difference in energy barriers between the conformational step and the chemical step could vary with the DNA substrate, pH, or viscosity (*10*). So under varied reaction conditions it is possible that the reverse "opening" rate could become more comparable to or even slower than the rate of chemistry. In light of the entire Pol β mechanism, the rate constant for the chemistry step (k_3) for both matched and mismatched dNTP incorporation is a significant term in the $k_{pol}/K_{d\ app}$ expression and, therefore, remains in the Pol β's fidelity expression:

$$\text{fidelity} = \frac{\left(\frac{k_{pol}}{K_{d\ app}}\right)_{cor}}{\left(\frac{k_{pol}}{K_{d\ app}}\right)_{inc}} = \frac{\left(\frac{k_1 k_2 k_3}{k_2 k_3 + k_{-1}(k_{-2} + k_3)}\right)_{cor}}{\left(\frac{k_1 k_2 k_3}{k_2 k_3 + k_{-1}(k_{-2} + k_3)}\right)_{inc}}$$

This expression can be further simplified upon assumption of the rapid ground state binding of the nucleotide ($k_{-1} \gg k_2$):

$$\text{fidelity} = \left(\frac{k_2^{cor}}{k_2^{inc}}\right)\left(\frac{k_3^{cor}}{k_3^{inc}}\right)\left(\frac{(k_{-2} + k_3)^{inc}}{(k_{-2} + k_3)^{cor}}\right)\left(\frac{K_d^{inc}}{K_d^{cor}}\right)$$

A recent comparative analysis of matched and mismatched dNTP incorporation pathways demonstrated similar forward rates of Pol β's conformational "closing" (*14*), such that the k_2^{cor}/k_2^{inc} term is close to unity and negligible in overall fidelity expression value. In contrast, difference in the magnitudes of the reverse "opening" rates suggests that the k_{-2} term diverges by a factor of 5 in the fidelity expression. The comparison of matched and mismatched dNTP incorporation suggests that the k_3^{cor}/k_3^{inc} is likely the largest term in Pol β's fidelity expression; however, the value of the K_d^{inc}/K_d^{cor} term (the ground state nucleotide binding) is also substantial (*14*). Therefore, in contrast to what has been reported for T7 DNA polymerase (*13*), incorrect dNTP discrimination by Pol β is realized at all three elementary steps (ground state binding, conformational step, and chemistry) with the major contribution from the rate-limiting chemistry step. We need to point out, however, that this "stepwise" discrimination does not serve as fidelity "enhancement". DNA polymerase fidelity is determined by the enzyme's differential ability to stabilize the highest energy transition state for matched versus mismatched dNTP, and the mismatched nucleotide discrimination at each step of the reaction pathway merely reflects this ability (*3*). Importantly, the mechanism presented in Scheme 3 does not include the catalytic Mg^{2+} binding step. This step may be significantly

perturbed for the mismatched incorporation and should be thoroughly examined in order to complete the kinetic mechanism of incorrect dNTP discrimination. Overall, the novel approaches for examination of Pol β's reverse conformational "opening" presented here contribute to the ongoing full evaluation of all of the microscopic rate constants in the polymerase kinetic pathway, which is a prerequisite for complete understanding of the mechanism of fidelity of DNA polymerases.

REFERENCES

1. Kunkel, T. A., and Bebenek, K. (2000) DNA replication fidelity. *Annu. Rev. Biochem. 69*, 497–529.
2. Showalter, A. K., Lamarche, B. J., Bakhtina, M., Su, M. I., Tang, K. H., and Tsai, M.-D. (2006) Mechanistic comparison of high-fidelity and error-prone DNA polymerases and ligases involved in DNA repair. *Chem. Rev. 106*, 340–360.
3. Showalter, A. K., and Tsai, M.-D. (2002) A reexamination of the nucleotide incorporation fidelity of DNA polymerases. *Biochemistry 41*, 10571–10576.
4. Dahlberg, M. E., and Benkovic, S. J. (1991) Kinetic mechanism of DNA polymerase I (Klenow fragment): Identification of a second conformational change and evaluation of the internal equilibrium constant. *Biochemistry 30*, 4835–4843.
5. Johnson, K. A. (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem. 62*, 685–713.
6. Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., and Benkovic, S. J. (1987) Kinetic mechanism of DNA polymerase I (Klenow). *Biochemistry 26*, 8410–8417.
7. Patel, S. S., Wong, I., and Johnson, K. A. (1991) Pre-steady-state kinetic analysis of processive DNA replication including complete characterization of an exonuclease-deficient mutant. *Biochemistry 30*, 511–525.
8. Doublie, S., Sawaya, M. R., and Ellenberger, T. (1999) An open and closed case for all polymerases. *Structure 7*, R31−R35.
9. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Crystal structures of human DNA polymerase beta complexed with gapped and nicked DNA: Evidence for an induced fit mechanism. *Biochemistry 36*, 11205–11215.
10. 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.
11. Rothwell, P. J., Mitaksov, V., and Waksman, G. (2005) Motions of the fingers subdomain of Klentaq1 are fast and not rate limiting: Implications for the molecular basis of fidelity in DNA polymerases. *Mol. Cell 19*, 345–355.
12. Joyce, C. M., Potapova, O., Delucia, A. M., Huang, X., Basu, V. P., and Grindley, N. D. (2008) Fingers-closing and other rapid conformational changes in DNA polymerase I (Klenow fragment) and their role in nucleotide selectivity. *Biochemistry 47*, 6103–6116.
13. Tsai, Y. C., and Johnson, K. A. (2006) A new paradigm for DNA polymerase specificity. *Biochemistry 45*, 9675–9687.
14. Roettger, M. P., Bakhtina, M., and Tsai, M.-D. (2008) Mismatched and matched dNTP incorporation by DNA polymerase beta proceed via analogous kinetic pathways. *Biochemistry 47*, 9718–9727.
15. Sucato, C. A., Upton, T. G., Kashemirov, B. A., Batra, V. K., Martinek, V., Xiang, Y., Beard, W. A., Pedersen, L. C., Wilson, S. H., McKenna, C. E., Florian, J., Warshel, A., and Goodman, M. F. (2007) Modifying the beta,gamma leaving-group bridging oxygen alters nucleotide incorporation efficiency, fidelity, and the catalytic mechanism of DNA polymerase beta. *Biochemistry 46*, 461–471.
16. Sucato, C. A., Upton, T. G., Kashemirov, B. A., Osuna, J., Oertell, K., Beard, W. A., Wilson, S. H., Florian, J., Warshel, A., McKenna, C. E., and Goodman, M. F. (2008) DNA polymerase beta fidelity: Halomethylene-modified leaving groups in pre-steady-state kinetic analysis reveal differences at the chemical transition state. *Biochemistry 47*, 870–879.
17. Lin, P., Batra, V. K., Pedersen, L. C., Beard, W. A., Wilson, S. H., and Pedersen, L. G. (2008) Incorrect nucleotide insertion at the active site of a G:A mismatch catalyzed by DNA polymerase beta. *Proc. Natl. Acad. Sci. U.S.A. 105*, 5670–5674.
18. Lin, P., Pedersen, L. C., Batra, V. K., Beard, W. A., Wilson, S. H., and Pedersen, L. G. (2006) Energy analysis of chemistry for correct insertion by DNA polymerase beta. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13294–13299.
19. Johnson, K. A. (2008) Role of induced fit in enzyme specificity: A molecular forward/reverse switch. *J. Biol. Chem. 283*, 26297–26301.
20. Dunlap, C. A., and Tsai, M.-D. (2002) Use of 2-aminopurine and tryptophan fluorescence as probes in kinetic analyses of DNA polymerase beta. *Biochemistry 41*, 11226–11235.
21. Zhong, X., Patel, S. S., Werneburg, B. G., and Tsai, M.-D. (1997) DNA polymerase beta: Multiple conformational changes in the mechanism of catalysis. *Biochemistry 36*, 11891–11900.
22. Bakhtina, M., Lee, S., Wang, Y., Dunlap, C., Lamarche, B., and Tsai, M. D. (2005) Use of viscogens, dNTPalphaS, and rhodium(III) as probes in stopped-flow experiments to obtain new evidence for the mechanism of catalysis by DNA polymerase beta. *Biochemistry 44*, 5177–5187.
23. Steitz, T. A. (1999) DNA polymerases: Structural diversity and common mechanisms. *J. Biol. Chem. 274*, 17395–17398.
24. Steitz, T. A., Smerdon, S. J., Jager, J., and Joyce, C. M. (1994) A unified polymerase mechanism for nonhomologous DNA and RNA polymerases. *Science 266*, 2022–2025.
25. Luo, G., Wang, M., Konigsberg, W. H., and Xie, X. S. (2007) Single-molecule and ensemble fluorescence assays for a functionally important conformational change in T7 DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A. 104*, 12610–12615.
26. Kumar, S., Bakhtina, M., and Tsai, M.-D. (2008) Altered order of substrate binding by DNA polymerase X from African swine fever virus. *Biochemistry 47*, 7875–7887.
27. Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. S., and Tsai, M.-D. (1996) DNA polymerase beta: Pre-steady-state kinetic analysis and roles of arginine-283 in catalysis and fidelity. *Biochemistry 35*, 7041–7050.
28. Tang, K. H., Niebuhr, M., Tung, C. S., Chan, H. C., Chou, C. C., and Tsai, M.-D. (2008) Mismatched dNTP incorporation by DNA polymerase beta does not proceed via globally different conformational pathways. *Nucleic Acids Res. 36*, 2948–2957.
29. Batra, V. K., Beard, W. A., Shock, D. D., Pedersen, L. C., and Wilson, S. H. (2008) Structures of DNA polymerase beta with active-site mismatches suggest a transient abasic site intermediate during misincorporation. *Mol. Cell 30*, 315–324.
30. Goodman, M. F., Keener, S., Guidotti, S., and Branscomb, E. W. (1983) On the enzymatic basis for mutagenesis by manganese. *J. Biol. Chem. 258*, 3469–3475.
31. Arnold, J. J., Gohara, D. W., and Cameron, C. E. (2004) Poliovirus RNA-dependent RNA polymerase (3Dpol): Pre-steady-state kinetic analysis of ribonucleotide incorporation in the presence of Mn^{2+}. *Biochemistry 43*, 5138–5148.
32. Pelletier, H., Sawaya, M. R., Wolfle, W., Wilson, S. H., and Kraut, J. (1996) A structural basis for metal ion mutagenicity and nucleotide selectivity in human DNA polymerase beta. *Biochemistry 35*, 12762–12777.
33. Bloom, L. B., Otto, M. R., Beechem, J. M., and Goodman, M. F. (1993) Influence of 5′-nearest neighbors on the insertion kinetics of the fluorescent nucleotide analog 2-aminopurine by Klenow fragment. *Biochemistry 32*, 11247–11258.
34. Lee, I., and Berdis, A. (2006) Fluorescent analysis of translesion DNA synthesis by using a novel, non-natural nucleotide analogue. *ChemBioChem 7*, 1990–1997.
35. Beard, W. A., and Wilson, S. H. (2006) Structure and mechanism of DNA polymerase beta. *Chem. Rev. 106*, 361–382.
36. 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.
37. Gavish, B., and Werber, M. M. (1979) Viscosity-dependent structural fluctuations in enzyme catalysis. *Biochemistry 18*, 1269–1275.
38. Ahn, J., Kraynov, V. S., Zhong, X., Werneburg, B. G., and Tsai, M.-D. (1998) DNA polymerase beta: Effects of gapped DNA substrates on dNTP specificity, fidelity, processivity, and conformational changes. *Biochem. J. 831*, 79–87.
39. Starcevic, D., Dalal, S., Jaeger, J., and Sweasy, J. B. (2005) The hydrophobic hinge region of rat DNA polymerase beta is critical for substrate binding pocket geometry. *J. Biol. Chem. 280*, 28388–28393.
40. 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 poly-

merase beta active site: Role of aspartic acid 256, arginine 254, and arginine 258 in nucleotidyl transfer. *Biochemistry 34*, 15934–15942.

41. Radhakrishnan, R., and Schlick, T. (2004) Orchestration of cooperative events in DNA synthesis and repair mechanism unraveled by transition path sampling of DNA polymerase beta's closing. *Proc. Natl. Acad. Sci. U.S.A. 101*, 5970–5975.
42. Yang, L., Beard, W. A., Wilson, S. H., Broyde, S., and Schlick, T. (2002) Polymerase beta simulations suggest that Arg258 rotation is a slow step rather than large subdomain motions per se. *J. Mol. Biol. 317*, 651–671.
43. DeLucia, A. M., Grindley, N. D., and Joyce, C. M. (2007) Conformational changes during normal and error-prone incorporation of nucleotides by a Y-family DNA polymerase detected by 2-aminopurine fluorescence. *Biochemistry 46*, 10790–10803.
44. Arndt, J. W., Gong, W., Zhong, X., Showalter, A. K., Liu, J., Dunlap, C. A., Lin, Z., Paxson, C., Tsai, M.-D., and Chan, M. K. (2001) Insight into the catalytic mechanism of DNA polymerase beta: Structures of intermediate complexes. *Biochemistry 40*, 5368–5375.
45. Purohit, V., Grindley, N. D., and Joyce, C. M. (2003) Use of 2-aminopurine fluorescence to examine conformational changes during nucleotide incorporation by DNA polymerase I (Klenow fragment). *Biochemistry 42*, 10200–10211.
46. Li, Y., and Tsai, M.-D. (1993) Phospholipase A2 engineering. 10. The aspartate.histidine catalytic diad also plays an important structural role. *J. Am. Chem. Soc. 115*, 8523–8526.
47. Katz, A. K., Glusker, J. P., Beebe, S. A., and Bock, C. W. (1996) Calcium ion coordination: A comparison with that of beryllium, magnesium, and zinc. *J. Am. Chem. Soc. 118*, 5752–5763.

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