A Unified Kinetic Mechanism Applicable to Multiple DNA Polymerases†,‡

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ABSTRACT: After extensive studies spanning over half a century, there is little consensus on the kinetic mechanism of DNA polymerases. Using stopped-flow fluorescence assays for mammalian DNA polymerase β (Pol β), we have previously identified a fast fluorescence transition corresponding to conformational closing, and a slow fluorescence transition matching the rate of single-nucleotide incorporation. Here, by varying pH and buffer viscosity, we have decoupled the rate of single-nucleotide incorporation from the rate of the slow fluorescence transition, thus confirming our previous hypothesis that this transition represents a conformational event after chemistry, likely subdomain reopening. Analysis of an R258A mutant indicates that rotation of the Arg258 side chain is not rate-limiting in the overall kinetic pathway of Pol β, yet is kinetically significant in subdomain reopening. We have extended our kinetic analyses to a high-fidelity polymerase, Klenow fragment (KF), and a low-fidelity polymerase, African swine fever virus DNA polymerase X (Pol X), and showed that they follow the same kinetic mechanism as Pol β, while differing in relative rates of single-nucleotide incorporation and the putative conformational reopening. Our data suggest that the kinetic mechanism of Pol β is not an exception among polymerases, and furthermore, its delineated kinetic mechanism lends itself as a platform for comparison of the kinetic properties of different DNA polymerases and their mutants.

DNA polymerases play a central role in the genomic stability of living organisms. The kinetic and structural basis of high-fidelity DNA replication by DNA polymerases has been the subject of extensive studies for several decades, but little common ground has been established—even the fundamental question of which step is rate-limiting (1). Based on small magnitudes of thio-effects for correct nucleotide incorporation, pioneering kinetic studies of Klenow fragment (KF) and T7 DNA polymerase concluded that a step other than chemistry must be rate-limiting (2, 3). At the same time, pulse-chase experiments suggested the existence of a conformationally closed ternary complex (E·DNA·dNTP) (3, 4). Collectively, the above findings led to the suggestion that DNA polymerases control their fidelity by an induced-fit mechanism consisting of a rate-limiting conformational change (5). Subsequently, structural studies showed a subdomain-closing conformational change upon Mg2NTP binding to the E·DNA binary complex, which led to the dogma that this “open-to-closed” conformational change is rate-limiting and therefore the major determinant of fidelity (6–8).

However, results of our subsequent kinetic and structural studies collectively indicate that the subdomain-closing conformational change of Pol β is faster than chemistry (9, 10). We then suggested that the overall experimental data for Pol β are most consistent with the chemical step being rate-limiting and thereby being a major determinant of fidelity (11). Recent reports from other labs have provided additional evidence that subdomain closing is a fast process for Pol β and other DNA polymerases (12–14). Despite this, many continue to believe that a slow conformational step, possibly involving subtle rearrangements of active site residues, occurs prior to chemistry (15–18). There have also been suggestions that even if Pol β indeed lacks a rate-limiting step prior to chemistry, it is likely an exception among polymerases (16, 19).

As shown in our earlier stopped-flow studies, single correct dNTP incorporation by Pol β results in a biphasic fluorescence change (10, 20, 21). Extensive kinetic and structural studies have indicated that the fast fluorescence phase originates from a Mg2NTP-induced conformational change, and that the rate of the slow fluorescence phase matches the rate of chemistry (9, 22). One of our previous approaches was the application of varying reaction buffer viscosity to elucidate the physical events (23, 24) corresponding to the biphasic change in fluorescence observed in stopped-flow assays (22). The selective influence of viscosity on the rate of the fast fluorescence transition led us to the conclusion that the step corresponding to the fast phase involves substantial spatial movements within the E·DNA·dNTP...
ternary complex, which supports our earlier interpretation that the fast fluorescence transition originates from the subdomain-closing conformational change. Since the slow fluorescence transition, which matches the rate of single-nucleotide incorporation, is not likely to result from phosphodiester bond formation directly, we hypothesized (11, 22) that it actually results from a conformational step after chemistry (possibly subdomain reopening), but that its rate is limited by the slower chemical step (the blue trace in Figure 1A). A minimal kinetic scheme, as shown in Figure 1, has been proposed to describe the catalytic process of Pol β, though not all steps have been measured independently. This paper describes the decoupling of chemistry and a postchemistry conformational step, allowing dissection of the three key steps in a single catalytic cycle (closing, chemistry, and reopening) for Pol β. The approach was also used to test the suggested role of R258 in the catalytic cycle as predicted by computational studies (25–27). Furthermore, our results suggest that both the high-fidelity KF and the low-fidelity ASFV Pol X follow the same kinetic pattern, though the relative rates of their chemistry and postchemistry conformational steps differ.

MATERIALS AND METHODS

Materials. Ultrapure dNTPs and G-25 microspin columns were from GE Healthcare. [γ-32P]ATP was from MP Biomedicals. T4 polynucleotide kinase was from New England BioLabs. Reverse phase C18 cartridges were from Waters. BSA was from Roche. Materials and reagents not listed here were of standard molecular biology grade.

Purification of Enzymes. Both the recombinant wild-type rat Pol β and the R258A mutant were overexpressed and purified as previously described from the BL21(DE3)-pLysS Escherichia coli strain (28). To generate the R258A mutant, site-directed mutagenesis was carried out with forward primer 5′-CCACACAGGAGAATCGATATCGCGTTGATCCCCAAGATCGATAC-3′ and reverse primer 5′-GTACTGATCTTTGGGGATTACGCGATATCGATTCTCCTGTGG-3′ using the QuikChange method (Stratagene). Successful mutagenesis was verified by both sequencing of the plasmid (OSU Plant Microbe Genomics Facility) and electrospray ionization mass spectrometry of the mutant protein (OSU Campus Chemical Instrument Center). ASFV Pol X was expressed and purified as described previously (29). The Klenow fragment D355A/E357A mutant with abolished 3′→5′ exonuclease activity was expressed and purified according to the procedure described by Joyce (30). The enzyme concentrations were determined by UV absorbance at 280 nm using extinction coefficients of 21 200 M⁻¹ cm⁻¹, 14 770 M⁻¹ cm⁻¹, and 55 300 M⁻¹ cm⁻¹ for Pol β and R258A, Pol X, and KF, respectively. 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 polyacrylamide/urea gels and desalted using a reverse phase C18 cartridge. Primer, template, and down-
stream primer (where applicable) were annealed in a 1.1:1:1.15 molar ratio, respectively. The single downstream primer utilized was 5'-phosphorylated. DNA primers used in the chemical quench experiments were 32P labeled at the 5'-end using T4 polynucleotide kinase and [γ-32P]ATP (4500 Ci/mol) according to the manufacturer’s protocol. The labeled primers were separated from unreacted ATP using a G-25 microspin column.

Kinetic Assays. Pol β buffers consisted of 70 mM BisTris/Tris, 10 mM MgCl2, 1 mM DTT, 10–35% (w/v) glycerol, with the ionic strength adjusted to 81 mM with KCl, and pH varied from 6.1 to 8.7 (adjusted at 37 °C). Pol X buffer consisted of 50 mM Tris-borate, 10 mM MgCl2, 140 mM KCl, 1 mM DTT, 100 μg/mL BSA, 5–40% (v/v) glycerol at pH 7.5 or 9.0. If not specified otherwise, Pol β and Pol X assays were performed at 37 °C. The KF assays were performed at 20 °C in a buffer consisting of 50 mM Tris, 10 mM MgCl2, 1 mM DTT, 0–40% (w/v) glycerol at pH 7.5, conditions similar to those of previous studies (2, 31).

Chemical Quench Experiments. A typical reaction was initiated by rapid mixing of two solutions in the assay buffers described above: (a) 400 nM 32P-labeled DNA substrate and 1 μM DNA polymerase, and (b) suitable correct dNTP. All R258A assays used a larger enzyme excess to account for the notable decrease in its DNA binding affinity compared to that of wild-type (32). Pol β and KF reactions were performed for time points ranging from 3 ms to 10 s using a rapid chemical quench instrument (KinTek Corp., State College, PA). Reactions were quenched with either 0.6 M EDTA or 3 M HCl. Pol X reactions were performed by manual mixing and were quenched with formamide after time points ranging from 10 to 300 s. Product formation was analyzed by denaturing PAGE, followed by autoradiography using a STORM 840 PhosphorImager and ImageQuant 5.0 software (GE Healthcare).

Stopped-Flow Fluorescence Assays. Experiments were performed on an Applied Photophysics SX 18MV stopped-flow apparatus. The excitation wavelength was 312 nm with a spectral bandpass of 4 nm. 2-Aminopurine (2-AP) emission was monitored using a 360 nm high pass filter (Corion). Reactions were initiated as described for the chemical quench. Typically, a minimum of 7 runs were performed and averaged.

Data Analysis. Chemical quench data were fit using Sigma Plot 9.0 (Jandel Scientific) to a single exponential equation:

\[ [\text{DNA}_{t+1}] = A(1 - e^{-k_{\text{quench}}t}) \]

where A is amplitude and \( k_{\text{quench}} \) is the observed rate constant of single-nucleotide incorporation.

Stopped-flow data were fit using Applied Photophysics software to a double exponential equation:

\[ \text{fluorescence} = A_1 e^{-k_{\text{fast}}t} + A_2 e^{-k_{\text{slow}}t} + C \]

where \( k_{\text{fast}} \) and \( k_{\text{slow}} \) are the rate constants for the fast phase and the slow phase of the fluorescence transition, respectively. \( A_1 \) and \( A_2 \) are the corresponding amplitudes, and C is an offset constant.

RESULTS

Dissection of the Chemical and the Subdomain-Reopening Steps of Pol β. To test our hypothesis that the slow fluorescence transition originates from a postchemistry conformational step, rate-limited by the slower chemistry, we attempted to create reaction conditions in which either the chemical step was selectively facilitated or the conformational steps were selectively slowed. First, we found that, as pH increased, the rate of the slow fluorescence phase \( (k_{\text{slow}}) \) significantly increased, while the rate of the fast fluorescence phase \( (k_{\text{fast}}) \) remained relatively unchanged (Figure 2A). The free energy diagram at high pH could thus be represented by the green trace in Figure 1A, where the energy barrier of the chemical step is lowered relative to that of the blue trace at neutral pH, and could become comparable to the energy barriers of the conformational steps.

Throughout the pH range studied, the rate of dNTP incorporation \( (k_{\text{quench}}) \) obtained from rapid chemical quench matches \( k_{\text{slow}} \) (Figure 2B and Table 2). However, unlike our previously published results showing that only the fast fluorescence phase is sensitive to altered solvent viscosity at pH 7.0 (22), we found that both the fast and slow fluorescence transitions were slowed down by increasing viscosity at higher pH (Figures 2C and 3A). Based upon this observation, we further predict that by increasing buffer viscosity (slowing down the conformational steps selectively) at high pH the chemical step will no longer be the slowest step under single-turnover conditions. Then, if the slowest step corresponds to a conformational step preceding chemistry, it is expected that the chemical quench rate of single-nucleotide incorporation will be sensitive to altered buffer viscosity. Alternatively, if the slow fluorescence transition corresponds to a conformational step after chemistry, the chemical quench rate should not be affected by altered viscosity. As shown in Figure 3B, the rate of single dNTP incorporation \( (k_{\text{quench}}) \) remained unaffected as glycerol concentration increased from 10% to 35% at pH 8.3, while both \( k_{\text{fast}} \) and \( k_{\text{slow}} \) decreased significantly under this condition (Figure 3A). These observations suggest the following important points: first, phosphodiester bond formation is not limited by a step that involves large spatial movements within the Pol β ternary complex; second, by analogy to the fast fluorescence transition (22), the slow fluorescence transition originates from a step that involves conformational changes; and third, this conformational step occurs after chemistry.

The situation observed under conditions of high pH and high viscosity can be represented by the red trace of Figure 1A, where the rate of chemistry becomes faster than the rate of the slow fluorescence transition. To further support the correspondence of the slow fluorescence transition to a step after chemistry in the Pol β reaction pathway, we performed two-nucleotide incorporation rapid chemical quench experiments under conditions of high pH and high viscosity. As expected, the rate of the first nucleotide incorporation was not affected by viscosity (nearly identical to single-nucleotide incorporation experiments in Figure 3B, thus not shown), whereas that of the second dNTP incorporation was limited by the rate of the slow fluorescence transition, and was sensitive to viscosity (Figure 3C).

To test the possibility that the slow fluorescence change originates from translocation of DNA rather than subdomain reopening, we performed a stopped-flow assay using a single-nucleotide gapped DNA substrate. We found that the slow fluorescence transition is also present under these conditions (Figure 4). Since the product of dNTP incorporation into
Figure 2: (A) pH dependence of Pol β catalyzed dATP incorporation into 19/36AP in stopped-flow assays. Reactions were initiated by rapid mixing of solution A (containing 1 μM Pol β and 400 nM 19/36AP DNA substrate) with solution B (containing 200 μM dATP) at 37 °C. Both solutions contained 10% glycerol. Assay buffer pH (from top to bottom) is 6.1 (red), 6.5 (blue), 7.0 (pink), 7.3 (green), 7.5 (purple), 8.0 (cyan), 8.3 (yellow), and 8.7 (orange). Kinetic curves were fit to a double exponential and yielded following rate constants: pH 6.1, $k_{\text{fast}} = 57.4 \pm 0.5 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.341 \pm 0.002 \text{ s}^{-1}$; pH 6.5, $k_{\text{fast}} = 50.7 \pm 0.4 \text{ s}^{-1}$ and $k_{\text{slow}} = 1.23 \pm 0.01 \text{ s}^{-1}$; pH 7.0, $k_{\text{fast}} = 45.4 \pm 0.3 \text{ s}^{-1}$ and $k_{\text{slow}} = 3.54 \pm 0.02 \text{ s}^{-1}$; pH 7.3, $k_{\text{fast}} = 47.9 \pm 0.5 \text{ s}^{-1}$ and $k_{\text{slow}} = 7.44 \pm 0.06 \text{ s}^{-1}$; pH 7.5, $k_{\text{fast}} = 47.3 \pm 0.5 \text{ s}^{-1}$ and $k_{\text{slow}} = 9.43 \pm 0.07 \text{ s}^{-1}$; pH 8.0, $k_{\text{fast}} = 48.8 \pm 0.6 \text{ s}^{-1}$ and $k_{\text{slow}} = 14.8 \pm 0.1 \text{ s}^{-1}$; pH 8.3, $k_{\text{fast}} = 56.0 \pm 0.7 \text{ s}^{-1}$ and $k_{\text{slow}} = 17.6 \pm 0.2 \text{ s}^{-1}$; pH 8.7, $k_{\text{fast}} = 56.6 \pm 0.7 \text{ s}^{-1}$ and $k_{\text{slow}} = 16.0 \pm 0.2 \text{ s}^{-1}$. (B) pH dependence of $k_{\text{slow}}$ (triangles) and $k_{\text{quench}}$ (circles) in stopped-flow and chemical quench assays of Pol β catalyzed dCTP incorporation into 18/35AP. Reactions were initiated by rapid mixing of solution A (containing 1 μM Pol β and 400 nM 18/35AP DNA substrate) with solution B (containing 800 μM dCTP) at 25 °C. Both solutions contained 10% glycerol. Assay buffer pH (from top to bottom) is 6.6 (red), 7.0 (green), 7.4 (blue), 7.8 (pink), 8.0 (purple), 8.3 (green), 8.6 (blue), 9.0 (pink), and 9.4 (purple). Kinetic curves were fit to a double exponential and yielded following rate constants: pH 6.6, $k_{\text{fast}} = 0.178 \pm 0.001 \text{ s}^{-1}$ and $k_{\text{slow}} = 45.4 \pm 0.4 \text{ s}^{-1}$; pH 7.0, $k_{\text{fast}} = 0.606 \pm 0.001 \text{ s}^{-1}$ and $k_{\text{slow}} = 9.43 \pm 0.07 \text{ s}^{-1}$; pH 7.4, $k_{\text{fast}} = 2.03 \pm 0.01 \text{ s}^{-1}$ and $k_{\text{slow}} = 1.59 \pm 0.18 \text{ s}^{-1}$; pH 7.6, $k_{\text{fast}} = 2.81 \pm 0.01 \text{ s}^{-1}$ and $k_{\text{slow}} = 2.71 \pm 0.13 \text{ s}^{-1}$; pH 8.0, $k_{\text{fast}} = 6.40 \pm 0.01 \text{ s}^{-1}$ and $k_{\text{slow}} = 5.95 \pm 0.20 \text{ s}^{-1}$; pH 8.4, $k_{\text{fast}} = 13.7 \pm 0.1 \text{ s}^{-1}$ and $k_{\text{slow}} = 16.8 \pm 0.6 \text{ s}^{-1}$; pH 8.8, $k_{\text{fast}} = 21.8 \pm 0.1 \text{ s}^{-1}$ and $k_{\text{slow}} = 20.5 \pm 0.7 \text{ s}^{-1}$. (C) Viscosity effect on Pol β catalyzed dATP and dCTP incorporation into 19/36AP and 18/35AP in stopped-flow and chemical quench assays. Reactions were initiated by rapid mixing of solution A (containing 1 μM Pol β and 400 nM 19/36AP DNA substrate) with solution B (containing 800 μM dATP) at 37 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{\text{slow}}$ (s$^{-1}$)</th>
<th>$k_{\text{quench}}$ (s$^{-1}$)</th>
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<tbody>
<tr>
<td>18/35AP + dCTP$^a$</td>
<td>6.6 10.187 ± 0.001</td>
<td>0.181 ± 0.009</td>
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<tr>
<td>7.0 0.606 ± 0.001</td>
<td>0.569 ± 0.032</td>
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<tr>
<td>7.4 2.03 ± 0.01</td>
<td>1.59 ± 0.18</td>
<td></td>
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<tr>
<td>7.6 2.81 ± 0.01</td>
<td>2.71 ± 0.13</td>
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<tr>
<td>8.0 6.40 ± 0.01</td>
<td>5.95 ± 0.20</td>
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<tr>
<td>8.4 13.7 ± 0.1</td>
<td>16.8 ± 0.6</td>
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<tr>
<td>8.8 21.8 ± 0.1</td>
<td>20.5 ± 0.7</td>
<td></td>
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<tr>
<td>19/36AP + dATP$^a$</td>
<td>6.5 1.25 ± 0.01</td>
<td>1.24 ± 0.06</td>
</tr>
<tr>
<td>7.5 12.3 ± 0.1</td>
<td>11.6 ± 0.3</td>
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<tr>
<td>8.3 23.7 ± 0.5</td>
<td>26.9 ± 0.9</td>
<td></td>
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$^a$ Each reaction was initiated by rapid mixing of solution A (containing 1 μM Pol β and 400 nM 18/35AP DNA substrate) with solution B (containing 800 μM dCTP) at 25 °C. Each reaction was initiated by rapid mixing of solution A (containing 1 μM Pol β and 400 nM 19/36AP DNA substrate) with solution B (containing 800 μM dATP) at 37 °C.

Table 2: Stopped-Flow and Chemical Quench Rates for Pol β Catalyzed Single-Nucleotide Incorporation at Different pHs

single-nucleotide gapped DNA is nicked DNA, and in the crystal structure of Pol β with a nicked DNA substrate the enzyme has not translocated, it is unlikely that the slow phase of fluorescence change originates from the translocation of DNA to the next templating position.

R258A Mutant of Pol β Has a Facilitated Subdomain-Reopening Step. Computational studies suggested that re-orientation of R258 side chain is rate-limiting, and that the R258A mutant shows facilitated subdomain closing, consistent with a reported increased rate of nucleotide insertion (25, 27). Since our aforementioned results allowed the dissection of the chemical and the subdomain-reopening steps of wild-type Pol β, it was of further interest to employ similar techniques to examine the specific role of Arg258 in the conformational closing and reopening steps of the enzyme. To accomplish this, we performed kinetic analyses of the R258A mutant. As shown in Figure 5A, a stopped-flow trace for dATP incorporation into 19/36AP by R258A resembles that of wild-type. It was also confirmed by rapid chemical quench that the rate of single-nucleotide incorporation catalyzed by R258A ($k_{\text{quench}} = 13.6 \text{ s}^{-1}$, Figure 5A) is identical to that of WT ($k_{\text{quench}} = 13.7 \text{ s}^{-1}$, data not shown) and matches the rate of the slow fluorescence phase. If rotation of Arg258 is truly rate-limiting, a perturbation in $k_{\text{quench}}$ would be expected for this mutant.

To compare the rates of conformational changes between R258A and WT Pol β, both differential pH and viscosity were employed. Results show that at pH 7.5 $k_{\text{slow}}$ for R258A remained virtually unaltered upon increasing viscosity, whereas WT demonstrated a systematic decrease in its rate of slow fluorescence transition (Figure 5B). This result indicates that, for nearly the entire viscosity range examined at pH 7.5, the putative subdomain reopening step of R258A remains faster than chemistry, whereas for wild-type it becomes slower than chemistry, suggesting that the R258A mutant of Pol β has a facilitated subdomain-reopening step (the cyan trace of Figure 1B). Due to much faster chemistry at pH 8.3, the differences in the relative rates of subdomain reopening between the two enzymes diminished at this pH, and the fluorescence phases became comparably sensitive to viscosity (Figure 5B).

Analysis of the Kinetic Mechanism of Klenow Fragment. Dissection of the two steps in the Pol β reaction pathway
A double exponential fit yielded $k_{\text{fast}} = 86.9 \pm 4.7\text{ s}^{-1}$ and $k_{\text{slow}} = 13.2 \pm 0.2\text{ s}^{-1}$. The gray trace shows the corresponding experiment with dideoxy-terminated primer 19dd, where a single exponential fit yielded $k_{\text{fast}} = 49.1 \pm 3.4\text{ s}^{-1}$. Reactions were initiated by rapid mixing of solution A (containing 2 $\mu$M Pol $\beta$ and 800 nM DNA substrate) with solution B (containing 600 $\mu$M dATP). Reactions were performed at pH 7.7 and 37 °C in Pol $\beta$ assay buffer containing 10% glycerol.

The results described below suggest that the kinetic properties of KF at pH 7.5 resemble those of Pol $\beta$ under conditions of higher pH and higher viscosity. First, the rates obtained from chemical quench experiments are significantly faster than $k_{\text{slow}}$, but remain slower than $k_{\text{fast}}$ (Table 3). Second, both fast and slow fluorescence transitions were sensitive to viscosity (Figure 7A), similar to the behavior of Pol $\beta$ at pH 8.3 (Figure 2C). At the same time, the rate for single-nucleotide incorporation from chemical quench was relatively insensitive to viscosity (Table 3). Third, the result of the two-nucleotide incorporation experiment (performed with the 18/36AP DNA substrate in the presence of both dCTP and dATP) showed that formation of the two-nucleotide incorporation product was slower than the formation of the single-nucleotide incorporation product (Figure 2B). Furthermore, the rate of two-nucleotide incorporation matched the rate of the slow fluorescence transition as shown in Figures 6B and 7B. Similar results were obtained in a two-nucleotide incorporation experiment with dATP, dTTP, and 19/36AP (Table 3). These observations indicate that the slow fluorescence change corresponds to a physical step in KF reaction pathway which limits the rate of the second, but not the first dNTP incorporation. Based on these
data, we propose that the free energy diagram for KF is best represented by the red trace of Figure 1B. Similar to Pol β, the subdomain-closing step in the KF pathway is faster than chemistry. However, KF differs from Pol β in that the putative reopening of the closed conformation is slower than the chemical step under neutral pH conditions.

Comparison between Pol β and ASFV Pol X. ASFV Pol X is the smallest known polymerase and one of the lowest fidelity polymerases known (29). It shares high sequence and structural homology with Pol β, but lacks the thumb subdomain (33, 34). Its kinetic mechanism has not been studied by stopped-flow previously. As shown in Figure 8A, the stopped-flow fluorescence trace for Pol X catalyzed dCTP incorporation into 18/35AP DNA substrate by KF (black trace). A single exponential fit of the slow phase of fluorescence change yielded $k_{\text{slow}} = 21.3 \pm 0.08 \text{s}^{-1}$. The gray trace shows Mg2+CTP binding to the Pol β-18dd/36 binary complex. Each reaction was initiated by rapid mixing of solution A (containing 1 μM KF and 400 nM DNA substrate) with solution B (containing 60 μM of the corresponding correct dNTP). Reactions were performed at pH 7.5 and 20°C in KF assay buffer with no glycerol.

As in the case of Pol β, differential viscosity was applied to further support the similarity in Pol X and Pol β kinetic mechanisms, we showed that, like Pol β, incremental increases in pH for Pol X also resulted in corresponding increases in both stopped-flow slow fluorescence transition rates and chemical quench rates (Figure 8B).

incorporation from chemical quench ($k_{\text{quench}}$) matched $k_{\text{slow}}$ (Figure 8A). To further support the similarity in Pol X and Pol β kinetic mechanisms, we showed that, like Pol β, incremental increases in pH for Pol X also resulted in corresponding increases in both stopped-flow slow fluorescence transition rates and chemical quench rates (Figure 8B).

As in the case of Pol β, differential viscosity was applied to further support the assignment of the fast fluorescence transition to conformational closing. As shown in Figure 8C, $k_{\text{fast}}$ selectively decreased with increased viscosity, suggesting that the step corresponding to the fast fluorescence transition involves a conformational change in the Pol X-DNA-dNTP complex. At the same time $k_{\text{slow}}$ remained relatively unchanged (Figure 8C and Table 4). Interestingly, unlike Pol β, similar trends were observed for Pol X at pH 7.5 and 9.0 (Figure 8C), indicating that chemistry is the rate-limiting step for Pol X even at high pH. This, in turn, suggests that the relative difference between the rates of the rate-limiting chemical step and the putative conformational reopening is larger for Pol X than for Pol β (the lilac trace in Figure 1B).

Figure 5: (A) R258A mutant of Pol β stopped-flow and chemical quench overlay of dATP incorporation into 19/36AP. Reactions were initiated by rapid mixing of solution A (containing 4 μM R258A and 400 nM 19/36AP DNA substrate) with solution B (containing 800 μM dATP). Reactions were performed at pH 7.7 and 37°C in Pol β assay buffer containing 10% glycerol. A double exponential fit of the stopped-flow trace (pink) yielded $k_{\text{fast}} = 167 \pm 0.9 \text{s}^{-1}$ and $k_{\text{slow}} = 12.3 \pm 0.1 \text{s}^{-1}$. Chemical quench data (open circles) were fit to a single exponential and yielded $k_{\text{quench}} = 13.6 \pm 0.5 \text{s}^{-1}$. (B) Relative effect of buffer viscosity in stopped-flow for R258A (shaded bars) compared with WT (solid bars) catalyzed dATP incorporation into 19/36AP at pH 7.5 (pink) and pH 8.3 (cyan). Bars show the normalized rates of the slow fluorescence transition ($k_{\text{slow}}$) vs % glycerol in the reaction. Reactions were initiated as described in panel (A), with solution A containing either 4 μM R258A or 1 μM Pol β. Both solutions A and B contained equal indicated concentrations of glycerol in the Pol β assay buffer.

Figure 6: (A) KF stopped-flow fluorescence assay of dATP incorporation into 19/36AP (black trace). A double exponential fit yielded rate constants $k_{\text{fast}} = 256 \pm 3.4 \text{s}^{-1}$ and $k_{\text{slow}} = 3.12 \pm 0.01 \text{s}^{-1}$. The gray trace shows the fluorescence change induced upon Mg2+ATP binding to the Pol β-19dd/36AP binary complex. (B) Stopped-flow fluorescence assay of dCTP incorporation into 18/36AP DNA substrate by KF (black trace). A single exponential fit of the slow phase of fluorescence change yielded $k_{\text{slow}} = 21.3 \pm 0.08 \text{s}^{-1}$. The gray trace shows Mg2+CTP binding to the Pol β-18dd/36 binary complex. Each reaction was initiated by rapid mixing of solution A (containing 1 μM KF and 400 nM DNA substrate) with solution B (containing 60 μM of the corresponding correct dNTP). Reactions were performed at pH 7.5 and 20°C in KF assay buffer with no glycerol.
**DISCUSSION**

Further advances in the Mechanism of Pol β. Our results have demonstrated the dissection of the chemical step and a conformational step following chemistry, which supports our previous hypothesis that the slow fluorescence transition results from the subdomain-reopening step (11, 22). Although the slow fluorescence transition could in principle reflect any step after chemistry (reopening, PPi release, or DNA repositioning), it more likely corresponds to the subdomain-reopening conformational change because (i) use of different fluorophores and DNA substrates reveals that the direction of the slow fluorescence phase is always opposite to the direction of the fast fluorescence phase (10); (ii) at basic pH, $k_{\text{slow}}$ displays a similar sensitivity to altered viscosity as $k_{\text{fast}}$; and (iii) the slow fluorescence transition is still present when a gapped DNA substrate is used, suggesting that it does not correspond to translocation of DNA to the next templating position. However, it cannot be ruled out that the DNA translocation event is coupled with the subdomain-reopening conformational change as suggested in recent structural studies of T7 RNA polymerase (35).

Even though the data presented here support the hypothesis that chemistry determines the rate of single-nucleotide incorporation in Pol β catalysis, pH dependence analysis indicates that the rates of the chemical and conformational steps begin to approach one another as pH is increased. Thus for conformational closing, $k_{\text{fast}}$ is larger than $k_{\text{quench}}$ by a factor of $100$ at pH $6.1$ and by only a factor of $3$ at pH $8.3$. Moreover, for the putative subdomain reopening, $k_{\text{slow}}$ is rate-limited by chemistry at pH $7.0$, but becomes slower than $k_{\text{quench}}$ by a factor of $3$ at pH $8.3$ and $35\%$ glycerol.

**Role of Arg258 in the Kinetic Mechanism of Pol β.** On the basis of crystal structures of Pol β binary (open) and ternary (closed) complexes, it is known that upon subdomain closure the Asp192···Arg258 salt bridge is disrupted, and the Arg258 side chain rotates to form a hydrogen bond with Tyr296. This activates the enzyme by freeing Asp192 to coordinate the two metal ions required for catalysis (8). Recent computational studies have suggested that Arg258 side-chain reorientation is likely the rate-limiting microscopic event during the course of Pol β’s subdomain closing before chemistry and reopening after chemistry (25, 26). Furthermore, on this basis it has been proposed that an R258A mutant may have a lower energy barrier for the subdomain-closing step, and thus a faster rate of single dNTP incorporation (25, 27).

Our kinetic analysis indicates that the R258A mutation does not perturb the rate of single-nucleotide incorporation as measured by chemical quench, which further reinforces our proposed kinetic scheme in which conformational closing is not the rate-limiting step in Pol β’s nucleotide incorporation pathway. On the other hand, our results clearly demonstrate that the R258A mutant does possess facilitated subdomain reopening compared to that of WT. Overall, these
FIGURE 8: (A) Pol X stopped-flow and chemical quench overlay of dCTP incorporation into 18/35AP DNA. Reactions were initiated by mixing of solution A (containing 1 μM PolX and 400 nM 18/35AP DNA substrate) with solution B (containing 1 mM dCTP). Reactions were performed at pH 7.5 and 37 °C in Pol X assay buffer containing 10% glycerol. A double exponential fit of the stopped-flow trace yielded the following rate constants: pH 7.5; pink, k_{fast} = 0.0236 s^{-1} and k_{slow} = 0.0027 s^{-1}; pH 9.0, k_{fast} = 0.004 s^{-1} and k_{slow} = 0.0002 s^{-1}. A single exponential fit of chemical quench data (open circles) yielded k_{quench} = 0.0257 ± 0.0003 s^{-1}. The dark blue trace shows the fluorescence change induced upon MgdCTP binding to the Pol X-18kd/35AP complex. (b) pH dependence of Pol X catalyzed dCTP incorporation into 25/45AP DNA in stopped-flow fluorescence assays. Reactions were initiated by mixing of solution A (containing 1 μM PolX and 400 nM 25/45AP DNA substrate) with solution B (containing 1 mM dCTP). Reactions were performed at 37 °C in Pol X assay buffer containing 10% glycerol. Assay buffer pH (from top to bottom) is 7.4 (red), 7.8 (pink), 8.6 (cyan), 9.0 (green), and 9.4 (blue). Kinetic curves were fit to a double exponential and yielded the following rate constants: pH 7.4, k_{fast} = 1.49 ± 0.01 s^{-1} and k_{slow} = 0.0282 ± 0.0002 s^{-1}; pH 7.8, k_{fast} = 1.45 ± 0.02 s^{-1} and k_{slow} = 0.0623 ± 0.0004 s^{-1}; pH 8.6, k_{fast} = 0.95 ± 0.02 s^{-1} and k_{slow} = 0.254 ± 0.002 s^{-1}; pH 9.0, k_{fast} = 1.44 ± 0.02 s^{-1} and k_{slow} = 0.536 ± 0.004 s^{-1}; pH 9.4, k_{fast} = 1.60 ± 0.02 s^{-1} and k_{slow} = 0.667 ± 0.005 s^{-1}. (c) Viscosity effect in stopped-flow for Pol X catalyzed dCTP incorporation into 18/35AP at pH 7.5 and pH 9.0. Bars show the normalized rates of the fast fluorescence transition (k_{fast}) and slow fluorescence transition (k_{slow}) vs % glycerol in the reaction buffer and are colored as follows: red, k_{fast} pH 7.5; pink, k_{fast} pH 7.5; blue, k_{fast} pH 9.0; cyan, k_{slow} pH 7.5; and blue, k_{slow} pH 9.0. Both solutions A and B contained equal indicated concentrations of glycerol in Pol X assay buffer.

Table 4: Stopped Flow and Chemical Quench Rates for Pol X Catalyzed dCTP Incorporation into 18/35AP DNA

<table>
<thead>
<tr>
<th>% glycerol</th>
<th>k_{fast} (s^{-1})</th>
<th>k_{slow} (s^{-1})</th>
<th>k_{quench} (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.97</td>
<td>0.0236</td>
<td>0.0257 ± 0.0003</td>
</tr>
<tr>
<td>15</td>
<td>3.15</td>
<td>0.0240</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.10</td>
<td>0.0209</td>
<td>0.0235 ± 0.0002</td>
</tr>
<tr>
<td>25</td>
<td>1.49</td>
<td>0.0227</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.946</td>
<td>0.0217</td>
<td>0.0218 ± 0.0008</td>
</tr>
<tr>
<td>35</td>
<td>0.608</td>
<td>0.0259</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.424</td>
<td>0.0272</td>
<td>0.0262 ± 0.0011</td>
</tr>
</tbody>
</table>

*Reactions were initiated by mixing of solution A (containing 1 μM Pol X and 400 nM 18/35AP DNA substrate) with solution B (containing 1 mM dCTP). Both solutions A and B contained equal indicated concentrations of glycerol in Pol X assay buffer (pH 7.5). All double exponential fits of the stopped-flow traces yielded <2% standard errors in the rate constants.

results significantly highlight the importance of the Arg258 residue in isolated subdomain conformational events during the course of Pol β nucleotide incorporation, while also suggesting that rotation of this residue is not rate-limiting in the overall kinetic pathway. Most significantly, our results demonstrate the usefulness of our kinetic scheme and experimental approaches in examining specific effects of other mutants (36, 37).

Comparison of the Kinetic Mechanism of Pol β and Other DNA Polymerases: KF and Pol X. In extending our experimental approach to KF, we find no evidence for the additional prechemistry rate-limiting conformational change that has been previously suggested for this enzyme (16, 17). Instead, our data indicate that the kinetic mechanism of KF resembles that of Pol β under conditions of high pH and high viscosity. Under normal conditions, chemistry remains slower than the prechemistry conformational change for both Pol β and KF. However, their similarities diverge after chemistry, as KF possesses a slower conformational step after chemistry, most likely subdomain reopening (the red trace of Figure 1B).

Our kinetic analysis suggests that, similar to Pol β, Pol X has a fast non-rate-limiting prechemistry conformational step. Since the structure of the Pol X·DNA·dNTP ternary complex is not yet published, the exact structural nature of this conformational change remains to be established. However, unlike Pol β, the chemical step of Pol X remains rate-limiting even when the rate of the chemical step is enhanced at pH 9.0, leading us to conclude that during catalysis the TS energy differences between the chemical step and the conformational steps are larger for Pol X than for Pol β.

Although our results show that, for all three polymerases studied, the chemical step is the slowest in the correct nucleotide incorporation pathway through phosphodiester bond formation, we do not suggest that the same will necessarily hold true for all other DNA polymerases. However, we believe that the kinetic scheme shown in Figure 1 and the approaches we have employed can be used to examine the mechanism of many other DNA polymerases, even though the relative rates of different steps may differ for each enzyme. It is also important to point out that our results in this paper do not address the general issue of how the fidelity of a DNA polymerase is controlled, only that the factor(s) which contribute to the fidelity of Pol β are also likely to contribute to the fidelity of KF and Pol X.
Still, it is useful and relevant to address recent developments in the issue of how DNA polymerases control the fidelity. Recently, the hypothesis that DNA polymerases realize alternative pathways for correct and incorrect nucleotide incorporations puts emphasis on the role of the conformational step in active misalignment of catalytic residues to prevent misincorporation (15, 38). Johnson and colleagues suggested that even though the chemical step could be a major determinant of the rate of single nucleotide incorporation, it would not contribute significantly to DNA polymerase fidelity if the reverse rate of the “open-to-closed” conformational change was slower than the forward rate of chemistry (38). Overall, as indicated in our recent review (1), a complete understanding of the mechanism of fidelity will require understanding of the kinetic mechanism and the structural mechanism, for both correct and mismatched dNTP incorporations.

Possible Explanations for the Differences between this Report and Previous Reports on KF. Even though the results and interpretations of our current KF studies may appear to contradict those of earlier reports, involving both pulse-chase/pulse-quench-and-thio-analogue experiments (2, 4), they can be reconciled. Results of pulse-chase/pulse-quench experiments are often considered to be convincing evidence for the existence of a rate-limiting conformational change prior to phosphodiester bond formation. In reality, while such results indirectly reveal the existence of a “closed” ternary complex, which in turn validates the existence of an “open-to-closed” conformational change prior to correct nucleotide incorporation, they do not suggest that the conformational change is rate-limiting. Pulse-chase/pulse-quench experiments with KF, performed by Benkovic’s group in 1991, indicated accumulation of the nucleotide bound enzyme species (4). This observation strongly supports the presence of a conformational closing step before phosphodiester bond formation. The fast fluorescence transition observed in our KF stopped-flow experiments likely corresponds to this conformational change. Results of pulse-chase and processive synthesis experiments with KF suggested the existence of a slow conformational step after chemistry, which limits the rate of processive polymerization (4). Results of our KF kinetic studies are surely consistent with this observation.

The suggestion that the aforementioned prechemistry conformational change is rate-limiting was based on a small magnitude of thio-effect that was reported by Benkovic and colleagues in 1987 (2). Use of dNTP analogues, in which a nonbridging oxygen on the α-phosphate was replaced with a sulfur atom (dNTPαS), resulted in an observed change in the rate of nucleotide incorporation that was smaller than predicted based on the rates of model reactions of phosphothioates in solution. The absence of a substantial thio-effect was interpreted as evidence that a step other than chemistry is rate-limiting. However, it is now commonly accepted that thio-effects must be interpreted with caution (11, 16, 30, 39, 40). For a particular enzymatic reaction, the magnitude of the “intrinsic thio-effect” (defined as the observed thio-effect when the chemical step is fully rate-limiting) may not be the same as the expected thio-effect for a nonenzymatic reaction. For instance, the intrinsic thio-effect for correct nucleotide incorporation catalyzed by Pol β is ca. 7 (22).

Our stopped-flow data do somehow conflict with a recent report of stopped-flow studies on KF (17). In contrast to this study, we were not able to observe a slow change of fluorescence when dideoxy-terminated DNA substrate was used. In our view, the previously reported slow fluorescence change might originate from DNA dynamics not related to the single-nucleotide incorporation pathway. In fact, the reported change is slower than the rate of dNTP incorporation. Additionally, its rate does not show hyperbolic dependence on dNTP concentration, as would be expected for a step induced by correct nucleotide binding. However, further studies are needed in order to reconcile this study with our results. It should also be mentioned that, in previous stopped-flow studies on KF, the slow fluorescence transition observed during single-nucleotide incorporation was interpreted to be rate-limited by a slow conformational step prior to chemistry (17, 31). In contrast, our experiments demonstrated that the rate of the slow fluorescence transition corresponds to the rate of the postchemistry conformational step.

Future Perspectives. While we have developed and applied a variety of methods to explore the kinetic mechanism of Pol β, our goal is not only to understand the mechanism of Pol β but also to extend the platform developed for Pol β in order to evaluate other DNA polymerases and their mutants. The kinetic scheme shown in Figure 1 is likely to be universal for DNA polymerases, even though the relative rates of microscopic steps could differ for each polymerase. Although comparison of the kinetic behavior among different DNA polymerases is not straightforward since varied reaction conditions are often used, our multifaceted approach has established a useful platform for such comparison. In addition, the kinetic scheme and mechanistic studies presented here set the stage for comparison with recent theoretical analyses (41–43), and for systematic kinetic analyses of mismatched dNTP incorporations, a topic which is crucial for developing a rigorous understanding of the mechanism of polymerase fidelity.

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REFERENCES


