DNA Structure and Aspartate 276 Influence Nucleotide Binding to Human DNA Polymerase β

IMPLICATION FOR THE IDENTITY OF THE RATE-LIMITING CONFORMATIONAL CHANGE*

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Structures of DNA polymerase (pol) β bound to single-nucleotide gapped DNA had revealed that the lyase and pol domains form a “doughnut-shaped” structure altering the dNTP binding pocket in a fashion that is not observed when bound to non-gapped DNA. We have investigated dNTP binding to pol β-DNA complexes employing steady-state and pre-steady-state kinetics. Although pol β has a kinetic scheme similar to other DNA polymerases, polymerization by pol β is limited by at least two partially rate-limiting steps: a conformational change after dNTP ground-state binding and product release. The equilibrium binding constant, Kd(dNTP), decreased and the insertion efficiency increased with a one-nucleotide gapped DNA substrate, as compared with non-gapped DNA. Valine substitution for Asp276, which interacts with the base of the incoming nucleotide, increased the binding affinity for the incoming nucleotide indicating that the negative charge contributed by Asp276 weakens binding and that an interaction between residue 276 with the incoming nucleotide occurs during ground-state binding. Since the interaction between Asp276 and the nascent base pair is observed only in the “closed” conformation of pol β, the increased free energy in ground-state binding for the mutant suggests that the subsequent rate-limiting conformational change is not the “open” to “closed” structural transition, but instead is triggered in the closed pol conformation.

DNA polymerase β possesses both the nucleotidytransferase activity to fill the one-nucleotide gap and lyase activity required to remove the dRP flap. The central role of pol β in BER has been established (1, 2), and, in the absence of pol β, alternate BER pathways have been reported (3, 4).

DNA polymerase β is an attractive model to study polymerase mechanisms employed to assure efficient and faithful DNA synthesis. Its small size and lack of accessory proteins has facilitated its biochemical, kinetic, and structural characterization. Although pol β appears to have evolved separately from other classes of DNA/RNA polymerases of known structure (5), it shares many general structural and mechanistic features. Each of the polymerases possesses a groove along a broad face of the enzyme where nucleic acid binds. The polymerase domain of these enzymes has been likened to a right hand and is composed of finger, palm, and thumb subdomains (6). Polymerases have at least two acidic residues in the palm subdomain that bind catalytically essential metals. The crystal structures of the substrate complexes of pol β (7, 8), T7 DNA polymerase (9), Klentaq DNA polymerase (10), and HIV-1 reverse transcriptase (11) indicate that the reactive groups (i.e. metals, dNTP, and template-primer) have a similar three-dimensional arrangement. These structures are consistent with a “two metal ion” mechanism for nucleotidyl transfer (12).

In general, pol β also utilizes a similar kinetic mechanism as most other DNA polymerases. Steady-state kinetic analyses indicate that pol β follows an ordered addition of substrates (13). Employing pre-steady-state kinetics, it has been shown that Escherichia coli Klenow fragment (14, 15), T4 DNA polymerase (15, 16), T7 DNA polymerase (17), HIV-1 reverse transcriptase (18), and pol β (19) utilize a two-step nucleotide binding mechanism. Initial dNTP binding to a pol-DNA complex places the nucleotide within the active site. A subsequent conformational change results in the alignment of the catalytic atoms and rapid chemistry. Binding of the correct nucleotide facilitates this conformational change, whereas binding of the incorrect nucleotide does not. This “induced-fit” mechanism is consistent with the numerous structural differences that are observed upon binding of a correct nucleotide (8). In particular, the carboxyl-terminal subdomain (residues 262–335) is observed to reposition itself after binding a correct dNTP by rotation about an axis, α-helix M, that positions α-helix N so that several side chains can interact with the nascent base pair in this “closed” conformation.

Replicative DNA polymerases and reverse transcriptases often have an intrinsically associated exonuclease or endonuclease activity that complements their fundamental nucleotidytransferase function. DNA polymerase β also has an associated accessory activity that complements its DNA synthesis step in BER. Mild proteolysis of pol β separates a 31-kDa fragment,
which possesses the nucleotidyltransferase activity, from an amino-terminal 8-kDa fragment (20). This smaller domain binds strongly to 5′-phosphate groups in gapped DNA (21) and possesses the dRP lyase activity that is needed for removal of the dRP flap remaining after AP endonuclease cleavage of the abasic site intermediate in BER (22, 23). In the absence of a downstream DNA strand (i.e. no DNA gap), a crystal structure of a pol β-DNA-ddNTP complex revealed that the 8-kDa domain does not interact with the DNA or the 31-kDa domain, but instead is positioned some distance from the 31-kDa domain (Fig. 1A). The crystal structure of pol β bound to one-nucleotide gapped DNA demonstrates that the 8-kDa domain binds to the 5′-phosphate in the DNA gap and interacts with the carboxyl terminus of the 31-kDa domain (Fig. 1A) (8). This results in a more compact, doughnut-shaped structure forming a channel with dimensions appropriate for dNTP diffusion (Fig. 1B) (24). Thus, this conformation of the 8-kDa domain is potentially significant due to its proximity to the nucleotide binding pocket of pol β. The experiments described here were performed to examine nucleotide binding to pol β and to ascertain the influence of a gapped DNA structure. Additionally, the role of Asp276 and α-helix N in dNTP binding was investigated by comparing the effect of valine substitution for Asp276 on nucleotide binding with alternate DNA substrates. Asp276 is observed to form van der Waals contact with the base of the incoming nucleotide triphosphate in the closed pol β ternary substrate complex. Employing steady-state and pre-steady-state kinetics, we examine nucleotide binding to pol β. The results indicate that residue 276 interactions with the incoming nucleotide influence ground-state nucleotide binding during template base recognition. The implication is that α-helix N and Asp276 influence ground-state binding in the closed conformation that occurs prior to the rate-limiting conformational change and that subdomain closure is very rapid and not rate-limiting.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ultrapure deoxynucleoside triphosphates, [γ-32P]ATP, [α-32P]dCTP, [α-thio]dCTP, and MicroSpin G-25 columns were from Amersham Pharmacia Biotech. DE-81 filters were from Whatman. A 34-mer oligonucleotide substrate containing a single nucleotide gap was prepared by annealing three gel-purified oligonucleotides (Oligos Etc., Wilsonville, OR) to create a gap at position 16. Each oligonucleotide was resuspended in 10 mM Tris·HCl, pH 7.4, and 1 mM EDTA and the concentration determined from their UV absorbance at 260 nm. The annealing reactions were carried out by incubating a solution of 10 μM primer with 11 μM each of downstream and template oligonucleotides at 90 °C for 2 min followed by slow cooling to room temperature. The sequence of the gapped DNA substrate was as follows: primer, 5′-GATCCCCGGGTAC-3′; downstream oligonucleotide, 5′-GATCCCGGGGTAC-3′; template, 3′-GACGCTACGCGGATCC-5′.

The upstream primer in each case was 5′-labeled with [γ-32P]ATP (specific activity = 6.6 × 10^6 dpm/pmol) using T4 polynucleotide kinase (New England Biolabs) and contaminating radioactive ATP was removed with a MicroSpin G-25 column. The downstream primer was synthesized with a 5′-phosphate. A non-gapped DNA substrate was prepared by omitting the downstream oligonucleotide. A nicked substrate was prepared by annealing a 16-mer primer, where the 15-mer contained a single nucleotide gap, to a 6-mer template strand (specific activity of 21,200 cpm/μM). The upstream primer was 5′-labeled with [γ-32P]ATP (specific activity = 6.6 × 10^6 dpm/pmol) using T4 polynucleotide kinase (New England Biolabs) and contaminating radioactive ATP was removed with a MicroSpin G-25 column. The downstream primer was synthesized with a 5′-phosphate. A non-gapped DNA substrate was prepared by omitting the downstream oligonucleotide. A nicked substrate was prepared by annealing a 16-mer primer, where the 15-mer primer had a dCMP added to the 3′-end, and downstream oligonucleotide with template as described above.

**Human recombinant pol β (wild type and D276V)** was overexpressed in *Escherichia coli* cells and purified as described (25). The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 21,200 cm⁻¹ M⁻¹ (26).

**Kinetic Assays**—Rapid-quench assays were performed at 37 °C using a KinTek Model RQF-3 rapid-quench-flow apparatus (KinTek Corp., Austin, TX). Unless noted otherwise, all concentrations refer to the final concentration after mixing. The final reaction mixture typically consisted of 50 mM tris·HCl, pH 7.2, 100 mM KC1, 100 μg/ml bovine serum albumin, 10% glycerol, and 5 mM MgCl₂. The final concentration of enzyme and substrates are given in the text or figure legends.

**Enzyme and substrate DNA** were preincubated for 1 min and rapidly mixed with ddCTP and MgCl₂ to initiate each reaction. After various time periods, reactions were stopped by the addition of 0.25 M EDTA. The quenched samples were mixed with an equal volume of formamide dye and the products separated on 12% denaturing polyacrylamide gels.
The dried gels were analyzed using a PhosphorImager (Molecular Dynamics) to quantitate product formation. Steady-state time courses were measured by manual mixing and quenching at 37 °C to determine steady-state constants (i.e. apparent $k_{\text{cat}}$ and $K_m$). Following the appropriate incubation period, individual reactions were quenched with EDTA and the quenched reaction mixtures were applied to DEAE cellulose filters (DE-81). The unincorporated ([$\alpha$-32P]dCTP) was removed, and radioactive nucleotide incorporation was quantitated as described previously (27).

**Pyrophosphorylation**—A nicked DNA substrate (300 nM) was preincubated with 1 nM pol $\beta$ in reaction buffer. The reaction was initiated by the addition of 3 mM pyrophosphate and 5 mM MgCl$_2$. The reaction was quenched at various time periods with EDTA.

**Data Analysis**—Time courses were fitted to appropriate equations by nonlinear least squares methods. These equations are described under "Results." Progress curves were also fitted to kinetic models with KinTekSim, which is based on the programs KINSIM (28) and FITSIM (29).

**RESULTS**

**Influence of DNA Structure on Steady-state Efficiency of dCTP Incorporation**—The efficiency of correct nucleotide (i.e. dCTP) incorporation by pol $\beta$ was dependent on the nature of the template-primer (Table I). DNA polymerase $\beta$ catalyzed single-nucleotide gap filling at a steady-state rate of 0.8 s$^{-1}$. A similar rate of polymerization was observed when pol $\beta$ was bound to non-gapped DNA (i.e. the downstream oligonucleotide was omitted from the annealing reaction). In contrast, the dCTP concentration dependence of DNA synthesis indicated that $K_{\text{m(app)}}$ with a single-nucleotide gap DNA substrate was 12-fold lower than for a substrate without a gap (Table I), suggesting that the enzyme might possibly have a higher affinity for nucleotide when bound to gapped DNA. As a result of the lower $K_m$, catalytic efficiency ($k_{\text{cat}}/K_{\text{m(app)}}$) was over an order of magnitude greater for the gapped DNA substrate assayed under steady-state conditions than for the non-gapped DNA substrate.

**Equilibrium Nucleotide Binding**—Since steady-state kinetic parameters are often the composite of several rate constants, we sought to determine the equilibrium constant ($K_e$) for nucleotide binding to pol $\beta$-DNA. Using rapid-mixing and quenching techniques, we measured pre-steady-state product formation. When a high concentration of pol $\beta$ is preincubated with excess single-nucleotide gapped DNA (DNA:Enzyme = 3) and rapidly mixed with a high concentration of the complementary nucleoside triphosphate (i.e. dCTP) and Mg$^{2+}$, product formation is biphasic. A rapid burst of product formation precedes a slower apparent linear rate (Fig. 2, dashed line). The biphasic nature of the time course indicates that a step following chemistry is rate-limiting during steady-state catalysis. The slow step has been attributed to dissociation of the extended pol-DNA complex (19). The rates of product formation observed in Fig. 2 indicate that in contrast to processive replicative polymerases, $k_{\text{pol}}$ is not considerably greater than $k_{\text{effDNA}}$ for pol $\beta$. Scheme I describes the basic kinetic mechanism for single-nucleotide incorporation utilized by all DNA polymerases that have been examined. Following an ordered binding of substrate DNA and dNTP, a conformational change ($k_{\text{pol}}$) precedes rapid chemistry (see below). Pyrophosphate release is rapid, but dissociation of the extended product DNA is usually slow and at least partially rate-limiting.

$$k_{\text{pol}} = k_{\text{pol}}(\text{dNTP} ) / K_{\text{d}} + [\text{dNTP}]$$

FITTING THE TIME COURSE IN Fig. 2 TO AN EQUATION WITH RISING EXponential AND LINEAR TERMS, AS SHOWN BY EQUATION 2,

$$\text{Product} = A[1 - e^{-kt}] + v_{\infty} t,$$

results in a burst rate constant ($k_{\text{pol}}$) of 12.3 s$^{-1}$ and an apparent linear rate ($v_{\infty}$) of 120 nm/s (dashed line). With this analysis, the burst amplitude represents the apparent active concentration of enzyme (40 nm) so that $k_{\text{pol}}$ ($v_{\infty} / K_{\text{m(app)}}$) would be 3.0 s$^{-1}$. However, since $k_{\text{pol}} / k_{\text{effDNA}}$ is not considerably greater than 1, this analysis is not appropriate (see "Results" for details). Assuming rapid ligand binding and an “active” enzyme fraction of 70%, the data can be fitted to the model outlined in Scheme I to derive $k_{\text{pol}}$ and $k_{\text{effDNA}}$, of 7.7 and 2.8 s$^{-1}$, respectively (solid line). The simulated time course also indicates that the steady-state phase is short-lived (i.e. time course is non-linear).

**TABLE I**

| DNA$^a$          | $k_{\text{cat}}$ | $K_{\text{m(app)}}$ | $k_{\text{cat}}/K_{\text{m}}$ |
|------------------|------------------|-----------------------|--------------------------------|
|                  | s$^{-1}$ | $\mu$M | (s$^{-1}$. $\mu$M)$^{-1}$ |
| Non-gapped       | 0.7 ± 0.1 | 2.36 ± 0.75 | 0.30 ± 0.11 |
| Gapped           | 0.8 ± 0.1 | 0.18 ± 0.02 | 4.44 ± 0.75 |

$^a$The gapped DNA substrate contains a one-nucleotide gap and was prepared as described under “Experimental Procedures.”

The gapped DNA substrate contains a one-nucleotide gap and was preincubated with 1 nM pol $\beta$ prior to addition of dCTP. Initial velocity data were fitted to the Michaelis equation by nonlinear least squares methods.

**FIG. 2.** Pre-steady-state incorporation of dCTP by DNA polymerase $\beta$. DNA polymerase $\beta$ (100 nM) was pre-incubated with 300 nM gapped DNA substrate prior to mixing with an equal volume of 50 mM dCTP and 5 mM MgCl$_2$ to initiate the reaction. Reactions were stopped at time intervals and the products isolated and quantified as described under “Experimental Procedures.” The time course of product formation appears to be biphasic with an initial rapid exponential phase followed by a linear phase. The dashed line represents the best fit to an equation (Equation 2 under “Results”) with rising exponential and linear terms. The observed rate constant of the burst phase was 12.3 s$^{-1}$ followed by an apparent linear rate ($v_{\infty}$) of 120 nm/s. With this analysis, the burst amplitude represents the apparent active concentration of enzyme (40 nm) so that $k_{\text{pol}}$ ($v_{\infty}/K_{\text{m(app)}}$) would be 3.0 s$^{-1}$. However, since $k_{\text{pol}} / k_{\text{effDNA}}$ is not considerably greater than 1, this analysis is not appropriate (see “Results” for details). Assuming rapid ligand binding and an “active” enzyme fraction of 70%, the data can be fitted to the model outlined in Scheme I to derive $k_{\text{pol}}$ and $k_{\text{effDNA}}$, of 7.7 and 2.8 s$^{-1}$, respectively (solid line). The simulated time course also indicates that the steady-state phase is short-lived (i.e. time course is non-linear).
The equilibrium binding affinity (K_d) of pol β-DNA complexes with dCTP was measured by single-turnover analysis as described under "Results."

| Enzyme       | DNA        | K_d(dCTP) | k_pol/K_d |
|--------------|------------|-----------|-----------|
| Wild-type    | Non-gapped | 5.1 ± 0.2 | 22.0 ± 3.6| 0.23 ± 0.04 |
| Wild-type    | Gapped     | 10.0 ± 0.8| 5.6 ± 2.0 | 1.79 ± 0.65 |
| D276V        | Non-gapped | 3.2 ± 0.2 | 6.1 ± 1.3 | 0.52 ± 0.11 |
| D276V        | Gapped     | 6.3 ± 0.9 | 0.6 ± 0.3 | 10.5 ± 5.5  |

a The gapped DNA substrate has a one-nucleotide gap and was prepared as described under "Experimental Procedures."
rapidly mixing an excess of dCTP (50 μM) and 5 mM MgCl₂. Reactions were quenched at varying times up to 1 s and the products isolated and quantified as outlined under “Experimental Procedures.” Time courses were fitted to Equation 2 to estimate the apparent amplitude. As discussed under “Results,” the amplitude is proportional to the concentration of pol-DNA complex. The amplitudes are plotted against the concentration of one-nucleotide gapped DNA. The quadratic equation (Equation 4) was fitted to the data to estimate an association rate constant of 8.6 × 10⁷ M⁻¹ s⁻¹ and in turn can be used to estimate a dissociation rate constant of 1.9 s⁻¹, which gives a Kd of 22 ± 13 nM and an apparent active fraction of enzyme of 70 ± 10 nM.

proportional to the concentration of pol-DNA complex, as discussed above (see Equation 3). To determine the equilibrium binding affinity of pol β for the gapped DNA substrate, a fixed concentration of pol β (pol₁ = 100 nM) was preincubated with increasing concentrations of substrate DNA (DNA₂ = 25–200 nM). The concentration of pol-DNA complex was estimated by rapidly mixing an excess of dCTP (50 μM) and measuring the time course of single-nucleotide incorporation. The time courses were biphasic and the amplitude of the burst phase increased as a function of increasing DNA concentration. The DNA concentration dependence of the burst amplitude is illustrated in Fig. 4 and fitted to the quadratic equation (Equation 4).

\[
[\text{pol-DNA}] = 0.5(K_d + \text{pol} + \text{DNA}) - 0.25(K_d + \text{pol} + \text{DNA})^2
\]

The K_d and maximum burst amplitude were 22 and 70 nM, respectively. From Equation 3 and kₚₒˡ (Fig. 3B and Table II), the maximum burst amplitude can be used to calculate kₚₒˡ(DNA₂). This results in a dissociation constant of 1.9 s⁻¹ and in turn can be used to estimate an association rate constant of 8.6 × 10⁷ M⁻¹ s⁻¹ (K_d = kₚₒˡ/Kₐᵦₕ₉₉₦).

Influence of DNA Gap on Nucleotide Affinity—As shown in Table I, pol β fills single-nucleotide gapped DNA substrates nearly 15 times more efficiently than non-gapped substrates under steady-state conditions. To determine if the binding affinity of the incoming nucleotide (i.e. Kᵦₕ₉₉₇₆) is influenced by the single-nucleotide gap, single-turnover analysis (pol/DNA = 5) was employed to quantify nucleotide binding to a non-gapped DNA substrate (the downstream primer was omitted). Using this non-gapped DNA substrate, pol β was found to bind and incorporate dCTP with Kᵦₕ₉₉₇₆ of 22 μΜ and kₚₒˡ of 5 s⁻¹ (Table II). This represents a 4-fold decrease in binding affinity as compared with a gapped DNA complex and an 8-fold decrease in insertion efficiency.

Role of Residue 276 in the Nucleotide Binding Pocket—The structure of the pol β-DNA-ddCTP complex reveals that there are van der Waals interactions between the base moiety of the incoming nucleotide and the Cβ of Asp²⁷⁶, Fig. 5 (7, 8). Steady-state kinetic analysis and cross-linking studies previously suggested that removal of the electron-negative side chain by valine or glycine substitution resulted in an apparent increase in binding affinity (32). To determine whether the binding affinity of the incoming nucleotide is altered by removing the negative charge, but retaining van der Waals interactions resulted in an apparent increase in binding affinity (32). To determine whether the binding affinity of the incoming nucleotide is altered by removing the negative charge, but retaining van der Waals interactions indicated that the Kᵦₕ₉₉₇₆ for the incoming nucleotide triphosphate decreased 3.6- and 9-fold, respectively, as compared with wild-type enzyme (Table II). Thus, the binding affinity for the incoming nucleotide is increased nearly 40-fold for the mutant enzyme when utilizing a gapped DNA substrate as compared with wild-type enzyme on a non-gapped DNA substrate.

Incorrect Nucleotide Binding—To investigate whether Asp²⁷⁶ participates in the binding of an “incorrect” nucleotide (e.g. dTTP), the binding affinity of thymidine triphosphate with a gapped DNA substrate was determined for wild-type pol β and the D276V mutant. For the kinetic mechanism described above (Scheme I), when kₚₒˡ becomes very slow (i.e. rate-limiting), then Kᵦₕ₉₉₇₆ is equivalent to Kᵦₕ₉₉₇₆ (33–35). DNA polymerases generally discriminate against incorrect nucleotides by binding them weakly and incorporating them slowly. Thus, incorporation of an incorrect nucleotide is always rate-limiting for polymerases that exhibit low processivity, where processivity is defined as a competition between nucleotide incorporation and polymerase dissociation from the template-primer (i.e. kₚₒˡ/Kᵦₙ₉₉₆). This analysis does not require that the active fraction of enzyme be known. When the dTTP concentration dependence of incorporation opposite the template deoxycytosine in the single-nucleotide gapped DNA substrate was examined, the Kᵦ₉₉₉₆ was 330 ± 60 and 130 ± 20 μM (five
DISCUSSION

Nucleic acid polymerases must select (bind and incorporate) the correct nucleotide from the many structurally similar molecules to ensure accurate and efficient nucleic acid synthesis. DNA polymerase binding of the incoming nucleoside triphosphate has been mechanistically and kinetically described as occurring in at least two steps. After initial complex formation, an isomerization of the polymerase ternary complex leads to a productive catalytic complex where chemistry occurs rapidly (Scheme II). Each of the steps illustrated in Scheme II: (ground-state binding, isomerization, chemistry) offers an opportunity for selection of the correct nucleotide.

![Scheme II](https://example.com/scheme.png)

**Scheme II**

The contribution of each of these steps to the accuracy of nucleotide selection for DNA synthesis depends on the specific DNA polymerase. Whereas polymerases generally bind the incorrect nucleotide weakly and incorporate (k_{pol}) them slowly (e.g., T7 DNA polymerase and HIV-1 reverse transcriptase), the Klenow fragment of *E. coli* pol I does not appear to utilize ground-state binding to increase selectivity (K_{d(incorrect)} \approx K_{d(correct)}) (36). The lack of a significant elemental effect when the non-bridging oxygen on the \( \alpha \)-phosphorus is substituted with sulfur is consistent with a rate-limiting conformational change in a two-step dNTP binding mechanism for human DNA polymerase \( \beta \). This interpretation is supported by fluorescence changes of the template base analogue 2-aminopurine that occur upon nucleotide binding (37). The environment of the templating base, as monitored by fluorescence, changes at a rate that is similar to nucleotide incorporation even when incorporation is prevented (i.e., no chemistry) by using a dideoxynucleotide-terminated primer. A similar approach had been used to separate chemical and non-chemical steps of nucleotide binding with Klenow fragment and T4 DNA polymerase (15).

DNA polymerase \( \beta \) utilizes ground-state binding to selectively incorporate dCTP opposite a templating deoxyguanine.

that becomes cross-linked to the polymerase (32). Additionally, crystal structures of binary pol-dNTP complexes reveal that the dNTP is bound in the active site through its triphosphate moiety (41–43). The triphosphate moiety, therefore, offers minimal specificity toward accuracy for correct nucleotide binding. The sugar also affords some specificity, since \( \alpha \)-deoxyribose is preferred over ribose in the presence of DNA. However, discrimination during dNTP binding originates mainly from the identity of the base and its hydrogen bonding capacity and steric complementarity with the templating base. Ground-state binding of a nucleotide offers the opportunity to check proper Watson-Crick hydrogen bonding and/or steric complementarity. Formation of a base pair with good geometry induces a conformational change that results in rapid incorporation of the nucleotide. Thus, the rate of insertion is limited by a conformational change that triggers chemistry. The identity in structural terms of this rate-limiting conformational change will be discussed here.

Several significant structural changes occur upon substrate (DNA and dNTP) binding to pol \( \beta \) and other DNA polymerases as inferred from comparison of structures of apoenzyme with substrate bound complexes. These conformational changes occur with both the polymerase and the substrate. The rate-limiting conformational change observed kinetically has been postulated to be the large subdomain movement that is observed by comparing the crystal structures of open pol and pol-DNA complexes with those of the closed ternary pol-DNA-dNTP complexes (see Ref. 44 for a review). For pol \( \beta \), in addition to significant movement of the 8-kDa lyase domain upon binding gapped DNA, the carboxyl-terminal subdomain is observed to “close” upon the exposed face of the nascent base pair. Rotation around an axis, \( \alpha \)-helix M, positions \( \alpha \)-helix N to probe correct Watson-Crick geometry (7, 8, 24). Superimposing the polymerase catalytic subdomains of the binary one-nucleotide gapped DNA substrate with that including the incoming dNTP (ternary complex) illustrates this subdomain movement (Fig. 6A) and reveals that the 8-kDa domain moves slightly to form an even more compact structure (not shown) (24). Numerous active site side chain and substrate motions are associated with these conformational differences. Assuming that valine substitution for Asp\(^{276}\) does not produce additional active site structural changes, the finding that interactions between Asp\(^{276}\) and the incoming dNTP influence ground-state dNTP binding implies that the conformational change limiting insertion during the first turnover may not involve movement of \( \alpha \)-helix N. This follows because C/\( \beta \) of Asp\(^{276}\) must be in close contact with the base of the incoming dNTP during template base annealing. The van der Waals interaction between C/\( \beta \) of Asp\(^{276}\) and the correct dNTP is observed in the closed conformation indicating that the closed conformation itself may trigger the rate-limiting conformational change. A “structural signal” to indicate that positioning of \( \alpha \)-helix N in the closed position has occurred could be transmitted to the catalytic metal-binding site through the altered positioning observed with Arg\(^{268}\), Glu\(^{269}\), Arg\(^{258}\), and Asp\(^{192}\) in the open and closed forms of pol \( \beta \) (Fig. 6B). From a comparison of open and closed forms of pol \( \beta \) ternary complexes, it has also been suggested that thumb closure repositions the mononucleotide binding motif (residues 179–189) closer to the primer 3'-hydroxyl (8).

DNA polymerase \( \beta \) is an integral component of the BER pathway. It is responsible for removing the 5'-dRP flap and filling the one-nucleotide gap generated during BER (for a review, see Refs. 45 and 46). DNA polymerase \( \beta \) processively fills short DNA gaps (= 5 nucleotides) where the downstream DNA is \( 5' \)-phosphorylated (23, 47). In contrast, DNA synthesis occurs in a distributive fashion with non-gapped DNA. This is
attributed to the 5'-phosphate binding activity of the amino-terminal 8-kDa domain (23). The 8-kDa domain interacts with the carboxyl-terminal domain when bound to a one-nucleotide DNA gap (Fig. 1A). This interaction buries approximately 130 Å² of surface area. In the closed ternary complex of the one-nucleotide gap DNA structure, this solvent-inaccessible interface increases to 300 Å².

We have examined the influence of the template-primer structure on nucleotide binding by pol β. Previous studies that examined the influence of template-primer structure on the catalytic efficiency of pol β have reported dissimilar effects. In one study, the catalytic efficiency was increased over 2500-fold when using a single-nucleotide gap, as compared with a non-gapped DNA substrate (48). In contrast, only a modest influence (10-fold) of gap structure on catalytic efficiency has also been reported (49). In the current study, the catalytic efficiency increased about 10-fold as determined from steady-state (Table I) or pre-steady-state kinetics (Table II) with a one-nucleotide DNA gap, as compared with non-gapped DNA. With a template-primer system that is typically employed for kinetic study of DNA polymerases (non-gapped DNA), pol β binds the correct nucleotide with a lower affinity than when bound to gapped DNA (Table II). This could be due to the role that the amino-terminal 8-kDa domain may play in forming the dNTP binding pocket. In the absence of downstream DNA (i.e. no gap), the dNTP binding site is “exposed” relative to that observed in the ternary complex with a one-nucleotide gapped DNA (Fig. 1). As illustrated in Fig. 1, this results in a doughnut-like structure that apparently gates dNTP binding through a cavity leading to the polymerase active site. This type of polymerase architec-

Fig. 6. Comparison of open and closed DNA polymerase β-substrate complexes. A, the catalytic subdomains of the binary pol-DNA complex (1bpx, blue-green) and ternary complex (+ddCTP; 1bpy, purple) were superimposed and indicate that rotation around helix M (about 30°) positions helix N next to the new base pair. The DNA backbone, templating base, and incoming ddCTP (closed ternary complex) are illustrated. Asp²⁷⁶ (D276) is illustrated in a ball-and-stick representation and stacks with the base of the incoming ddCTP in the closed complex. B, the position of α-helix N can be structurally transmitted to the catalytic metals through the altered interactions between Arg²⁷⁸ (R283), in helix N, and Asp²⁸² (D192) that coordinates both active site Mg²⁺ ions (A and B). This may occur through altered interaction observed between Glu²⁹⁵ (E295) and Arg²⁵⁸ (R258) in the open (inactive) and closed (active) forms of pol β. Phe²⁷² (F272) is postulated to transiently interfere with interactions between Asp²⁷⁶ and Arg²⁵⁸ permitting an interaction with Glu²⁹⁵. Any of these subtle structural movements inferred from comparison of the open and closed complexes could kinetically limit chemistry and represent the rate-limiting conformational change. This figure was made with Molscript (61) and rendered with Raster3D (62).

Fig. 7. Electrostatic surface potential of the dNTP binding pocket of pol β. The view is similar to that of Fig. 1. Blue and red represent positive and negative potentials, respectively. The template and gapped (primer and downstream DNA) strands are red and yellow, respectively. The incoming ddCTP (yellow) is situated in a cavity at the 3'-primer terminus. Asp²⁷⁶ (D276) contributes negative potential in the vicinity of the incoming ddCTP. It is surrounded by several basic residues (e.g. Lys²⁷ (K27), Arg⁴⁰ (R40), and Lys²⁸⁰ (K280)). This figure was made with GRASP (60) and Molscript (61) and rendered with Raster3D (62).
The low processivity of pol β makes it difficult to accurately analyze the biphasic time courses of product formation when DNA is in excess by pre-steady-state methods (enzyme/DNA < 1; Fig. 2). This is because $k_{\text{pol}}$ and $k_{\text{eff}}$ are both partially rate-limiting, and product inhibition makes it difficult to estimate the linear steady-state rate of product formation. Processivity is kinetically defined as the probability of inserting a nucleotide (i.e., $k_{\text{in}}$) to that of dissociating from the DNA substrate (i.e., $k_{\text{off}}$). Thus, with a one-nucleotide gapped DNA substrate the processivity of DNA polymerase $\beta$ is modest ($k_{\text{pol}}/k_{\text{off}} = 10^{-5}$ S$^{-1}$). This is consistent with the qualitative gel assay that had indicated pol $\beta$ can processively fill short gaps of up to 6 nucleotides (47). Single-turnover analysis (enzyme/DNA $\gg$ 1) of dNTP binding to DNA polymerase $\beta$ eliminates enzyme cycling. From the nucleotide-dependent exponential time courses, the ground-state binding affinity for dCTP and $k_{\text{pol}}$ for the wild-type and D276V polymerases were determined (Fig. 3; Table II). The amplitude of the exponential time courses should be equivalent to the concentration of DNA. Under the conditions of the assay, 95% of the DNA should be enzyme-bound. For several determinations, the amplitude of the rapid-exponential phase was $63 \pm 8\%$ and $37 \pm 6\%$ of the extendable DNA for the wild-type and D276V polymerases, respectively. This indicates that a population of these polymerases can bind in a nonproductive conformation that is sensitive to the valine substitution at Asp$^{276}$. To determine whether this nonproductive DNA binding form could isomerize to a productive complex without dissociating from DNA, unlabeled DNA was added to trap enzyme that may dissociate during the course of the reaction. The excess non-radioactively labeled DNA trap had no effect on the amplitudes or rates of the rapid or slow phases, indicating that for the wild-type enzyme that the nonproductive DNA binding form can isomerize to the productive form without dissociating from the DNA. Similar results were observed with the mutant enzyme except that the slow phase was partially sensitive to the added trap. A thorough kinetic and thermodynamic analysis of these alternate binding modes is currently under study.

The alternate binding modes result in a decreased population of polymerase poised for nucleotide insertion. Suo and Johnson (57, 58) observed similar kinetic behavior when HIV-1 reverse transcriptase binds templates with secondary structure and suggested that the template nucleic acid is blocking access to the pol dNTP binding pocket. A similar proposal for pol $\beta$ suggests that the DNA substrate may bind at the polymerase active site such that the terminal template-primer base pair is sitting where the nascent base pair would form. In this conformation the template-primer is out of register for DNA synthesis and would block an incoming nucleotide from entering the polymerase active site. This conformation is observed in the binary complex of pol $\beta$ bound to nicked DNA. In addition, the ability of DNA polymerases to catalyze pyrophosphorolysis requires that the terminal primer nucleotide move into the polymerase active site to achieve PP$_i$ addition. As the valine substitution at Asp$^{276}$ stabilizes the incoming nucleotide sitting in the active site, it also may stabilize the terminal primer nucleotide in the polymerase active site. This is consistent with the lower apparent active concentration of the mutant polymerase.

In conclusion, the dNTP binding affinity is increased for pol $\beta$ with a one-nucleotide gapped DNA substrate, suggesting a role for the amino-terminal lyase domain in nucleotide binding. Interestingly, altering the interactions with the incoming nucleotide and/or lyase domain by site-directed mutagenesis of Asp$^{276}$ also increased the binding affinity of the correct incoming nucleotide. This is the first example that we are aware of where strategic modification of the dNTP binding pocket has resulted in an increased nucleotide binding affinity. It will be interesting to determine if the observed changes in binding affinity are due to “ionic tethering” (59) where Asp$^{276}$ and Arg$^{40}$ modulate domain interactions to influence ligand binding. Finally, since this interaction between Asp$^{276}$ and the incoming dNTP is observed only in the closed conformation of pol $\beta$, the increase in the free energy in ground-state binding for the D276V mutant suggests that the rate-limiting conformational change is not the open to closed structural transition, but instead is triggered in the closed polymerase conformation. Deoxynucleoside triphosphate binding, therefore, occurs in at least three steps. First, there is an initial nonspecific binding that occurs primarily through polymerase interactions with the triphosphate/metal and sugar moieties of the nucleotide. This step is weak so as to facilitate rapid sampling of the nucleoside triphosphate pools and is kinetically silent. This sampling is probably associated with the subdomain movements inferred from the crystal structure differences between the binary pol-DNA and ternary substrate complexes. The second step,
ground-state binding, is associated with template base recognition through hydrogen bonding and/or steric complementarity. In turn, the degree of complementarity influences the probability of triggering a rate-limiting conformational change (i.e. step three) that leads to a catalytically competent complex that induces chemistry.

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