NMR Determination of Lysine pK_a Values in the Pol λ Lyase Domain: Mechanistic Implications

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ABSTRACT: The base excision repair (BER) process requires removal of an abasic deoxyribose-5-phosphate group, a catalytic activity that has been demonstrated for the N-terminal 8 kDa domain of DNA polymerase β (Pol β), and for the homologous domain of DNA polymerase λ (Pol λ). Previous studies have demonstrated that this activity results from formation of a Schiff base adduct of the abasic deoxyribose C-1′ with a lysine residue (K312 in the case of Pol λ), followed by a β-elimination reaction. To better understand the underlying chemistry, we have determined pK_a values for the lysine residues in the Pol λ lyase domain labeled with [ϵ-^{13}C]lysine. At neutral pH, the H_ϵ protons on 3 of the 10 lysine residues in this domain, K287, K291, and K312, exhibit chemical shift inequivalence that results from immobilization of the lysyl side chains. For K287 and K291, this results from the K287−E261 and K291−E298 salt bridge interactions, while for K312, immobilization apparently results from steric and hydrogen-bonding interactions that constrain the position of the lysine side chain. The pK_a value of K312 is depressed to 9.58, a value indicating that at physiological pH K312 will exist predominantly in the protonated form. Titration of the domain with hairpin DNA containing a 5′-tetrahydrofuran terminus to model the abasic site produced shifts of the labeled lysine resonances that were in fast exchange but appeared to be complete at a stoichiometry of ∼1:1.3, consistent with a dissociation constant of ∼1 μM. The ϵ-proton shifts of K273 were the most sensitive to the addition of the DNA, apparently due to changes in the relative orientation between K273 and W274 in the DNA complex. The average pK_a values increased by 0.55, consistent with the formation of some DNA−lysine salt bridges and with the general pH increase expected to result from a reduction in the net positive charge of the complex. A general increase in the Hill coefficients observed in the complex is consistent with the screening of the interacting lysine residues by the DNA. The pK_a of K312 residue increased to 10.58 in the complex, probably due to salt bridge formation with the 5′-phosphate group of the DNA. The pK_a values obtained for the lyase domain of Pol λ in the present study are consistent with recent crystallographic studies of Pol β complexed with 5-phosphorylated abasic sugar analogues in nicked DNA which reveal an open site with no obvious interactions that would significantly depress the pK value for the active site lysine residue. It is suggested that due to the heterogeneity of the damaged DNA substrates with which Pol λ as well as other related polymerases may be required to bind, the unexpectedly poor optimization of the lyase catalytic site may reflect a compromise of flexibility with catalytic efficiency.

Removal of the abasic flap from the 5′-end of a gapped DNA molecule, the deoxyribose-5-phosphate lyase (dRP lyase) reaction, is an essential and generally rate-limiting step in the base excision repair (BER) of damaged DNA (*1*−*4*). The repair enzyme DNA polymerase β, as well as the more recently discovered polymerase λ homolog, are able to catalyze both the polymerization and lyase steps of the BER process (*2*, *5*). The cytotoxic effect of inhibitors that target the dRP lyase activity of Pol β[1] is consistent with the idea that the unprocessed 5′-dRP residue is a detrimental BER intermediate (*6*, *7*). It has been shown that the lyase activity originates within the 8 kDa domains of these two proteins (*5*, *8*−*12*). Matsumoto and Kim (*9*) reported that the dRP lyase activity of the isolated pol β lyase domain was comparable to that of the intact enzyme, while Prasad et al. have found that the k_{cat} value for the isolated 8 kD domain was slightly greater than that of the intact enzyme, while the Michaelis constant was significantly higher, consistent with poorer binding of the gapped DNA substrate to the isolated domain than to the intact enzyme (*12*). Studies of site-directed mutants have demonstrated that K72 in the case of Pol β (*10*−*13*) and the structurally homologous K312 in the case of Pol λ (*5*) are critical for dRP lyase activity. The isolated 8 kDa domains from both polymerases retain their structure and have been characterized by NMR spectroscopy (*14*−*16*). Crystallographic structures are available for the full length Pol β enzyme and for a construct that includes the two corresponding (polymerase and lyase) domains in Pol λ (*17*, *18*). These "lyase" domains must be considered unusual

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[1] Abbreviations: BER, base excision repair; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; NMR, nuclear magnetic resonance; Pol β, DNA polymerase β; Pol λ, DNA polymerase λ.

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by normal enzyme standards. They are globular, α-helical structures that lack a well-developed active site binding cleft, although it has been suggested that helices A, B, and D form a binding groove in which the dRP lyase reaction takes place (*8*). A β-elimination mechanism for the lyase activity has been demonstrated by product analysis (*9*, *19*) and reductive trapping of a Schiff base intermediate (*13*). Crystal structures of the complexes formed between Pol β and nicked DNA in which the 5′-abasic sugar was replaced with reduced cyclic (tetrahydrofuran) or acyclic analogues have recently been determined (*20*).

Although the observation of a Schiff base intermediate is consistent with the proposal that the lyase reaction proceeds via a β-elimination rather than a hydrolysis reaction, the mechanistic details of the reaction are unclear. The recent determination of the structure of the Pol λ lyase domain allows structural comparisons between the corresponding domains of Pol λ and Pol β that have mechanistic implications (*14*). In particular, it becomes possible to evaluate proposed roles for structurally homologous residues which differ between the two enzymes. For example, it has been suggested that residue K35 or K60 in Pol β could play a role in the protonation of the oxygen in the deoxyribofuranose ring, creating the open-chain aldehyde form as a precursor to Schiff base formation (*8*). However, in Pol λ, the structurally homologous residues are arginines R275 and R308, which would not be expected to fulfill such a role, due to the much higher arginine pK_a. Similarly, a proposed catalytic role for H34 in Pol β (*21*) could not be fulfilled by the W274 structural homologue in Pol λ (*14*). Consequently, either the catalytic activities of the homologous domains follow somewhat different mechanisms or an alternative mechanism valid for both domains must be identified.

Lysine residues involved in Schiff base formation typically exhibit decreased pK_a values (*22*). Lowering of the lysine pK_a for optimal Schiff base formation has been observed in acetoacetate decarboxylase, which catalyzes the decarboxylation of acetoacetate to yield acetone and CO_2. In this case, a decrease of >4 pH units in the pK_a of K116 results primarily from its proximity to K115 (*23*). Given the high density of lysine and arginine residues in the Pol λ lyase domain (*14*, *17*), a similar mechanism could be operative for lowering the pK_a of K312. Alternatively, the catalytic lysine residues involved in Schiff base formation in a series of antibody aldolases show lowered pK_a values of 5.5−6.0 due to their hydrophobic environment (*24*). To gain more insight into the catalytic mechanism of the Pol λ lyase domain, we have determined the pK_a values of [ϵ-^{13}C]lysine-labeled Pol λ lyase domain using NMR spectroscopy. NMR spectroscopic methods are uniquely suited to the determination of individual pK values for all of the resolvable residues in a protein.

MATERIALS AND METHODS

Expression and Purification of Pol λ Lyase Domain. The 87 residue constructs used in these studies correspond to residues A242−H327 with an additional N-terminal methionine residue. [ϵ-^{13}C]Lysine-labeled lyase domain was prepared by growth on a medium containing unlabeled amino acids, except lysine, and 0.42 g/L of [ϵ-^{13}C] lysine, based on the medium composition described by Muchmore et al. (*25*). The domain was purified as described previously (*14*).

NMR Sample Preparation. NMR samples typically contained 0.15 mM Pol λ lyase domain in 90% H_2O and 10% ^2H_2O (v/v), with 100 mM KCl, 2 mM deuterated DTT, 0.1% NaN_3 (w/v), and 50 μM DSS, which served as a chemical shift standard. The pH of the solution was carefully adjusted by adding aliquots of concentrated HCl or KOH. The pH of the sample at room temperature was measured both before and after the NMR data were collected, and the average reading was used for data analysis. The pH meter was calibrated with commercial standard pH buffers 4.0, 7.0, and 10.0.

NMR Experiments and Data Processing. 2D ^{1}H−^{13}C HSQC spectra of the aliphatic carbon region were collected on a Varian 800 MHz Unity Inova spectrometer at 298 K, using a 5 mm Varian ^{1}H{^{13}C,^{15}N} triple-resonance probe with actively shielded *z*-axis gradients. The NMR data were processed using NMRPipe (*26*), and the spectra were analyzed using NMRView (*27*).

Data Fitting. Titration data were fit using the nonlinear least-squares fitting routine in *Mathematica* (Wolfram Research Inc., Champaign, Il) to the relation

$$\delta_{obs} = \delta_U + \frac{\Delta\delta}{1 + 10^{n(pK-pH)}} \quad (1)$$

where δ_U is the shift of the unprotonated lysine resonance, $\Delta\delta$ is the titration shift resulting from protonation of a group with the pK value indicated in the equation, and n is the Hill coefficient, which has sometimes been introduced in order to account for long-range interactions between titrable groups (*28*, *29*). For the three-parameter fit, δ_U, $\Delta\delta$, and pK were determined with n set equal to 1.0. Titrations involving two well-separated pK values were fit using the relation

$$\delta_{obs} = \delta_U + \frac{\Delta_1}{1 + 10^{pK_1-pH}} + \frac{\Delta_2}{1 + 10^{pK_2-pH}} \quad (2)$$

where δ_U is the low pH shift, Δ_1 is the shift contribution from a group with pK_1, and Δ_2 is the shift contribution due to titration of the group with pK_2.

Obtaining a meaningful error analysis of the pK_a determination is a nontrivial problem. Since the primary systematic error results from a failure to obtain a high pH asymptote of the titration curve, uncertainty in $\Delta\delta$ is the primary source of experimental error. To estimate this effect, we reanalyzed the data for each series of lysine titrations with a two-parameter fit, in which the value of $\Delta\delta$ was set equal to the value measured for N_α-acetyl lysine. The underlying rationale is that the various local contributions to the lysine shift will be small relative to the ^{13}C shift resulting from titration of the lysine amino group. An RMSD value for each series was then calculated as the root-mean-square difference between the pK_a values obtained using eq 1 and the analogous two-parameter fit:

$$RMSD = \sqrt{\frac{1}{N}\sum_{i}^{N}(pK - pK_2)^2} \quad (3)$$

RESULTS

The construct of the Pol λ lyase domain used in these studies spanning residues A242−H327 corresponds to the 8

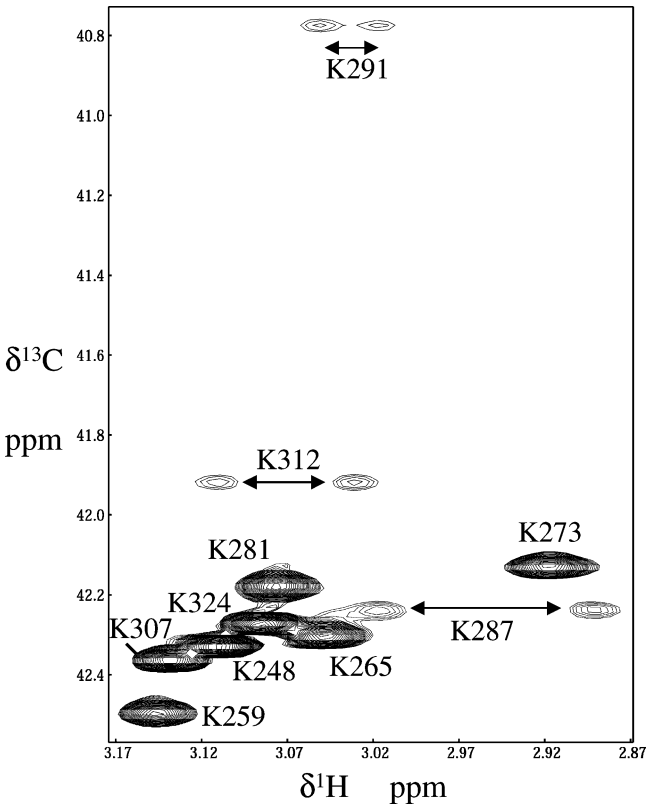

FIGURE 1: $^1H-^{13}C$ HSQC spectrum of the region containing the C_ϵ resonances of lysine in [ϵ-^{13}C]lysine-labeled Pol λ lyase domain. The spectrum was obtained by using Varian's gChsqc sequence on a Varian INOVA 800 MHz spectrometer at 298 K, with 256 × 842 complex points and acquisition times of 107 and 79 ms in t_1 and t_2, respectively. Eight scans were acquired per increment, with a 1.2 s delay between scans.

kDa domain used previously for structural characterization (*14*) and contains 10 lysine residues. The $^1H-^{13}C$ HSQC spectrum of [ϵ-^{13}C]lysine labeled Pol λ lyase domain obtained at neutral pH shows that, for seven of the lysine residues, the H_ϵ resonances are magnetically equivalent, while the remaining three residues have inequivalent H_ϵ resonances (Figure 1). Resonance assignments were obtained from our previous study (*14*), except for the K291 resonance, which was not previously assigned, probably due to its unusual ^{13}C shift and weak intensity. In general, the prochiral H_ϵ protons on lysine are predicted to be magnetically inequivalent due to the chirality of the protein; however, since they are located relatively far from the chiral centers and are highly dynamic, no significant shift inequivalence is generally observed. Thus, only lysine side chains which are significantly immobilized will experience sufficient magnetic inequivalence to produce observable shift inequivalence toward the end of the side chain. The physical basis for the inequivalent shifts for the H_ϵ protons of K287 and K291 is immediately apparent from an examination of the crystal structure (PDB code: 1RZT) (*17*). Both of these residues are involved in salt bridges: K287−E261 ($N_\zeta-O_{\epsilon 2}$ = 2.72 Å) and K291−E298 ($N_\zeta-O_{\epsilon 2}$ = 3.47 Å), which will lead to significant immobilization of the side chain, consistent with the observed $H_{\epsilon 1}/H_{\epsilon 2}$ resonance inequivalence (Figure 2). The basis of the proton shift inequivalence for K312 is somewhat less obvious. From the 1RZT crystal structure, the K312 amino group is positioned 3.70 Å from the Y279 hydroxyl oxygen and 3.71 Å from the R308 carbonyl oxygen, suggesting that H-bonding interactions may be primarily responsible for the relative immobilization of the K312 side chain. The amino group is also 5.25 Å from $O_{\epsilon 1}$ on E311, so that there also may be some degree of electrostatic interaction between these residues. In addition, the side chain of K312 is less exposed to the solvent and, hence, more constrained by packing interactions with other residues, compared with the other lysines in the domain. Residues making van der Waals contact with K312 include L260, L263, Y279, M309, and E315. A solvent accessibility calculation based on the 1NZP NMR structure (*14*) also reveals K312 to have the lowest accessibility.

Table 1: Three-Parameter Fit of Titration Data[a]

	lyase domain			lyase domain−DNA complex			
residue	pK^b	$\Delta\delta$	slope $\Delta(^1H)/\Delta(^{13}C)$	pK^c	$\Delta\delta$	slope $\Delta(^1H)/\Delta(^{13}C)$	H_ϵ inequiv?
K248	10.27	0.68	−0.45	10.50	0.84	−0.42	
K259	10.07	0.73	−0.41	10.49	1.02	−0.40	
K265	10.23	0.60	−0.38	10.92	1.68	−0.31	
K273	10.07	0.84	−0.43	10.48	1.77	+0.15	
K281	10.01	1.01	−0.34	10.54	1.12	−0.30	
K287	10.65	1.90	−0.17[d]	10.58	1.14	−0.18	yes
K291	10.26	0.88	−0.20	11.03	4.02	−0.12	yes
K307	10.16	0.28	−0.94	11.07	0.72	−0.98	
K312	9.58	0.57	−0.32	10.58	1.09	−0.39	yes
K324	10.27	0.43	−0.35[d]	10.77	0.83	−0.40	
av	10.16	0.79	−0.40	10.71	1.42	−0.335	
N_α-acetyllysine	10.80	1.05	−0.39				

[a] Data were fit to eq 1, with δ_U, $\Delta\delta$, and pK allowed to vary and n set equal to 1.0. [b] RMSD from eq 3: ±0.41. [c] RMSD from eq 3, with data for K273 and K291 omitted: ±0.16. [d] In a few cases for which plots of $\delta(^1H)/\delta(^{13}C)$ showed significant nonlinearity, three initial pH points were removed and the $\Delta(^1H)/\Delta(^{13}C)$ slope redetermined. In all cases, the change in slope was minimal (see the Supporting Information).

In addition to producing magnetic inequivalence, the salt bridge interactions of K287, K291, and perhaps K312 would be expected to result in an elevation of the corresponding pK_a values. As discussed below, K287 exhibits the highest pK_a value and the value for K291 is above the mean, while K312 exhibits the lowest pK_a, consistent with the lack of a significant electrostatic interaction with E311 (Table 1).

The pK_a values for the 10 lysine residues in our construct obtained from a 3-parameter fit of the ^{13}C titration data to eq 1 are summarized in Table 1, and typical titration fits for four residues, K259, K291, K307, and K312, are shown in Figure 3. The ^{13}C shifts for lysine C_ϵ were used in preference to the ^1H shifts due to their larger magnitude and, consequently, the reduction in the relative perturbations that conformational changes and other titrating residues would introduce into the data. Determination of individual pK_a values for protein residues is a fundamentally problematic issue for several reasons: (1) pH variation results in multiple residue titrations which, at least in principle, can all contribute to the shift observed for any particular nucleus; (2) conformational changes typically accompany residue titrations; (3) the range over which the pH can be varied without causing the protein to unfold is typically limited, so that good asymptotic chemical shift values often are not readily obtained. The use of an additional parameter, the Hill coefficient, has been proposed as a basis for evaluating the

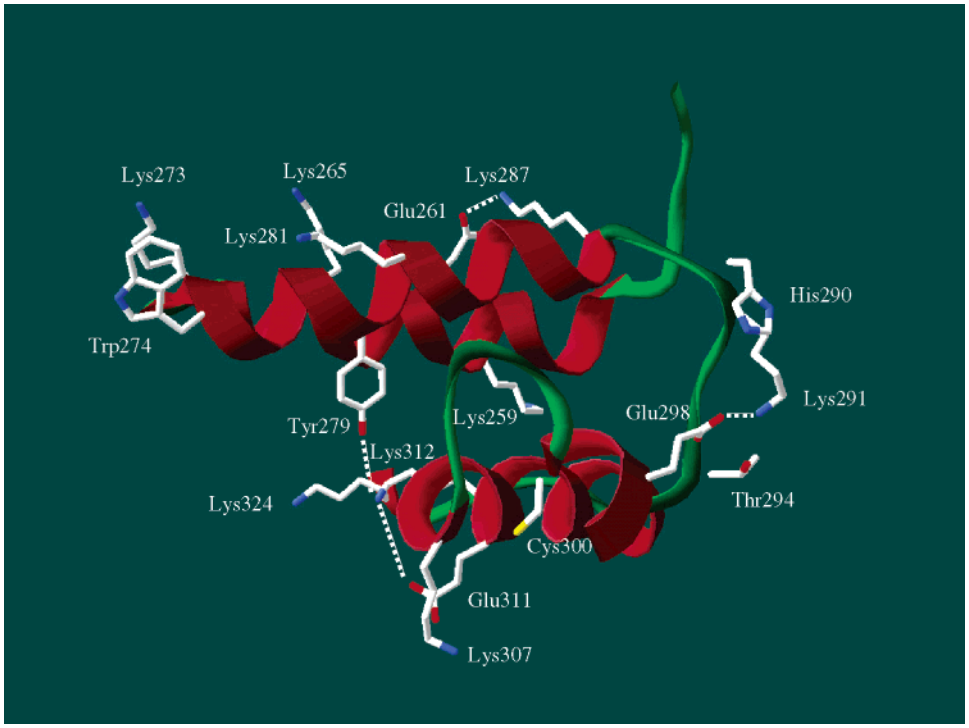

FIGURE 2: Crystal structure of the lyase domain of DNA Pol λ showing the positions of 9 of the 10 lysine residues with some of the interactions that immobilize the side chains of K287, K291, and K312. Although the construct begins at S245, K248 is not visible due to disorder of the N-terminus in the crystal structure. The K312 side chain is largely obscured in this view; a more revealing view of K312 is shown in Figure 6 (based on structure 1RZT) (*17*).

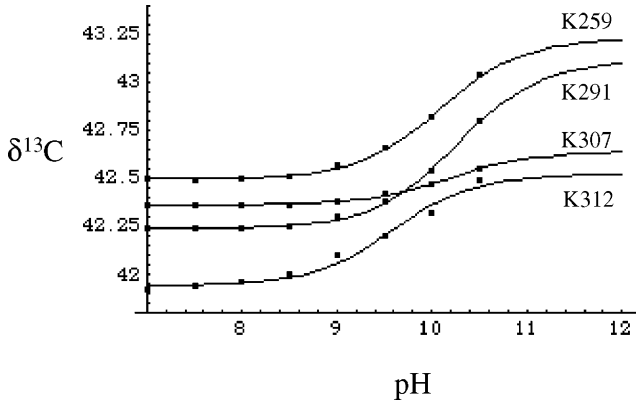

FIGURE 3: Titration data for K259, K291, K307, and K312. The curves are the three-parameter fits. Titration fits for only four residues are shown in order to limit the congestion of the figure.

Table 2: Hill Coefficient Analysis[a]

	lyase domain			lyase domain−DNA complex		
residue	pK^a	$\Delta\delta$	Hill coeff	pK^a	$\Delta\delta$	Hill coeff
K248	10.56	0.91	0.82	10.49	0.83	1.02
K259	10.29	0.91	0.82	10.45	0.97	1.05
K265	10.43	0.75	0.86	11.24	2.48	0.89
K273	10.39	1.15	0.76	10.37	1.57	1.15
K281	10.34	1.40	0.74	10.28	0.85	1.46
K287	10.68	2.04	0.99	10.53	1.08	1.05
K291	10.60	1.24	0.80	10.69	2.60	1.22
K307	10.46	0.37	0.80	10.78	0.49	1.16
K312	10.32	1.07	0.50	10.50	1.00	1.08
K324	10.68	0.71	0.70	10.67	0.74	1.08
av	10.47	1.06	0.78	10.60	1.26	1.11

[a] For the fits in this table, all four parameters, δ_U, $\Delta\delta$, pK, and n, were allowed to vary.

significance of interactions between titrable residues (*28*, *29*). The electrostatic interaction between a pair of titrating lysine residues has the effect of lowering the measured pK_a value and extending the titration curve, leading to a Hill coefficient <1, while positive cooperativity results in a Hill coefficient >1. However, titration of nearby groups can also directly contribute to the shift of an observed nucleus, independent of the effect on the pK_a of the titrating residue. The problem of obtaining a meaningful Hill coefficient in the absence of a good asymptotic titration limit was noted by Laurents et al. (*28*). Analysis of the ^{13}C shift data using a four-parameter relation that included a Hill coefficient increased the average lysine pK_a value by 0.29 (Table 2). This analysis yields a significantly higher pK_a value for K312, increasing from 9.58 to 10.32, along with a Hill coefficient of 0.5, the lowest value for any of the lysine residues. The unusually low Hill coefficient obtained for K312 may result from a strong interaction with the closely positioned Y239 residue. Thus, simultaneous deprotonation of Y239 as the pH increases tends to favor a protonated K312 in order to form a salt bridge, and it therefore requires a higher pH to fully deprotonate K312. This flattens out the titration curve, leading to a fit with Hill coefficient <1.0. The pK_a values for residues K287 and K291, which are involved in salt bridge interactions, are among the highest (Table 2). K324 also exhibits a high pK_a; however, the curvature of the $\delta(^1H)$ vs $\delta(^{13}C)$ plot (Figure 4) makes the interpretation of this value subject to additional uncertainty.

The introduction of two-dimensional methods that allow both the ^{1}H and ^{13}C shifts for particular residues to be determined in parallel represents an important advance for the analysis of protein titration data. If the shifts of both the lysine ϵ-carbon and ϵ-protons are dominated by the titration of the ϵ-amino group, a plot of the ^{1}H vs the ^{13}C shifts should be linear. Protein conformational changes which are driven

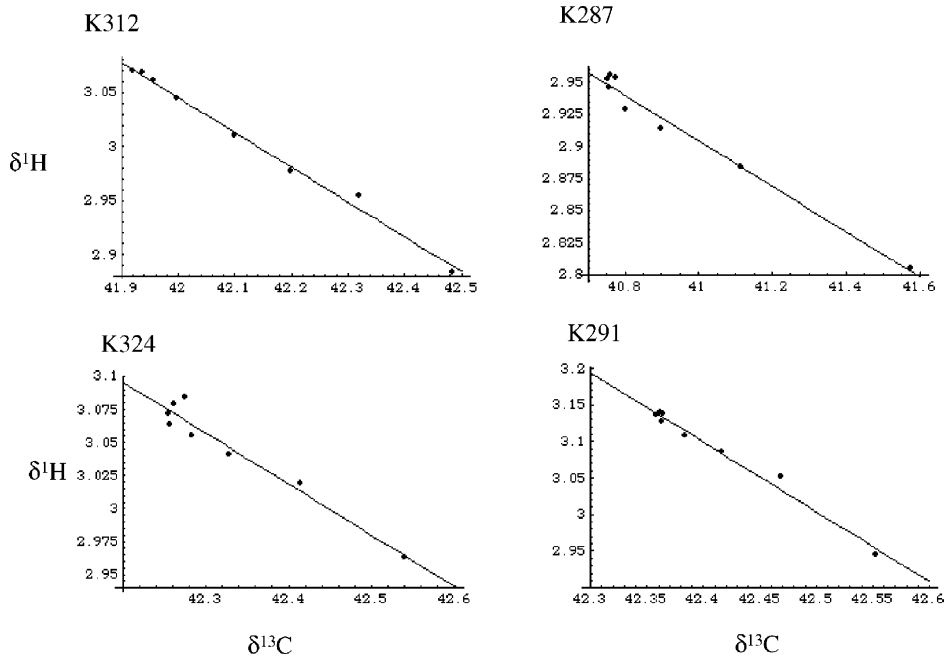

FIGURE 4: Plots of $\delta(^1H)$ vs $\delta(^{13}C)$ for residues K312, K298, K324, and K291. The plots span the pH range from 7.0 to 10.5.

by and accompany the titration of the lysine residue will contribute to the ^1H and ^{13}C shifts to a different extent than titration of the ϵ-amino group, so that the slope of the $\Delta(^1H)/\Delta(^{13}C)$ line will in general be altered. Alternatively, if the titration of other residues with different pK_a values contributes significantly to either the ^1H or ^{13}C shifts of the observed residue, then the $\delta(^1H)$ vs $\delta(^{13}C)$ plot will exhibit curvature. Plots of $\delta(^1H)$ vs $\delta(^{13}C)$ for the catalytically significant K312 residue and for the three residues showing the most significant deviation from linearity, K287, K291, and K324, are shown in Figure 4. The $\Delta(^1H)/\Delta(^{13}C)$ slopes for residues K287 and K324 were redetermined after dropping the initial, nonlinear points and gave very similar values (Supporting Information). The nonlinearity observed for K291 may result from titration of the closely positioned H290 residue (Figure 2). Similarly, the shifts of K324 will be influenced by titration of H327 and Y267 (Y267 OH to K324 C_ϵ distance of 3.11 Å). The physical basis for the nonlinearity of the K287 plot is not obvious and may be indicative of a local conformational change that is sensitive to a lower pK_a titration. The slopes of the $\delta(^1H)$ vs $\delta(^{13}C)$ plots for the two lysine residues that are involved in salt bridges, K287 and K291, are unusually low, while the slope for K307 is unusually high, as is the titration shift $\Delta\delta$ (Table 1). The basis for the last effect is unclear.

Effect of DNA Binding. Since the active complex of the domain contains bound DNA, it was important to determine the effect of DNA on the lysine pK_a values. For this study, we utilized a short segment with the sequence 5′-p-XGGC-GAAGCCA-3′, based on the compact 7-nucleotide hairpin originally identified by Hirao et al. (*30*): 5′-GCGAAGC-3′. The abasic site, labeled "X" above, was replaced by a more stable tetrahydrofuran ring. The expected base-pairing interactions, shown in the inset in Figure 5, were consistent with ^1H spectra of the imino protons of the hairpin DNA (data not shown). For this study there was no need to use a more complex DNA ligand, such as a gapped substrate, since at best the primer strand would not interact with the lyase domain and at worst it would complex with a second lyase

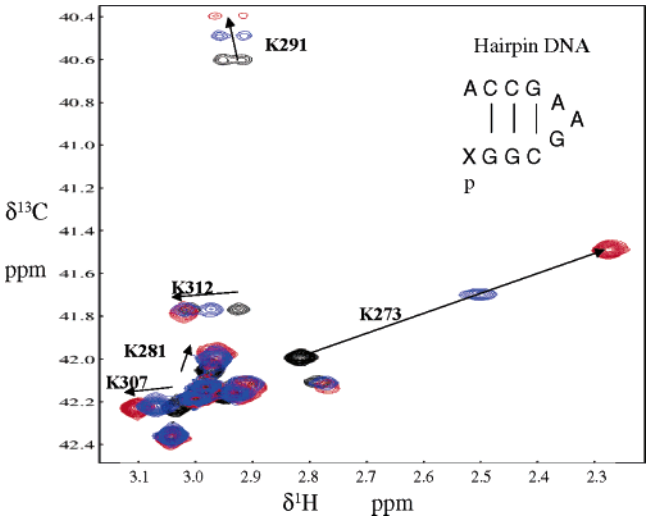

FIGURE 5: $^1H-^{13}C$ HSQC spectra of the region containing the C_ϵ resonances of lysine in [ϵ-^{13}C]lysine Pol λ lyase domain complexed with the hairpin DNA shown in the inset. The protein to DNA ratios were 1:0 (black), 1:0.5 (blue), and 1:1.3 (red). NMR parameters are as in Figure 1. The sample temperature was 25 °C.

domain (see the Supporting Information). The anticipated binding interaction with Pol λ would position the 3′-terminal adenosine in the templating base position, but in the complex formed with the isolated lyase domain the 3′-terminal nucleotide probably is relatively mobile. Fromme and Verdine (*31*) compared the crystallographic structures of the related endonuclease III containing either a trapped Schiff base adduct or a tetrahydrofuran mimic of the abasic site and found them to be virtually identical. Hence, this complex should provide a useful model of the interaction with the abasic site.

The superposed $^1H-^{13}C$ HSQC spectra obtained at protein to DNA ratios of 1:0, 1:0.5, and 1:1.3 are shown in Figure 5. As is apparent from the figure, complexation of the lyase domain with the DNA leads to large ^1H and ^{13}C shifts for K273 and to smaller ^{13}C shifts for K281 and K291. Lysine residues K307 and K312 are shifted primarily in the ^1H

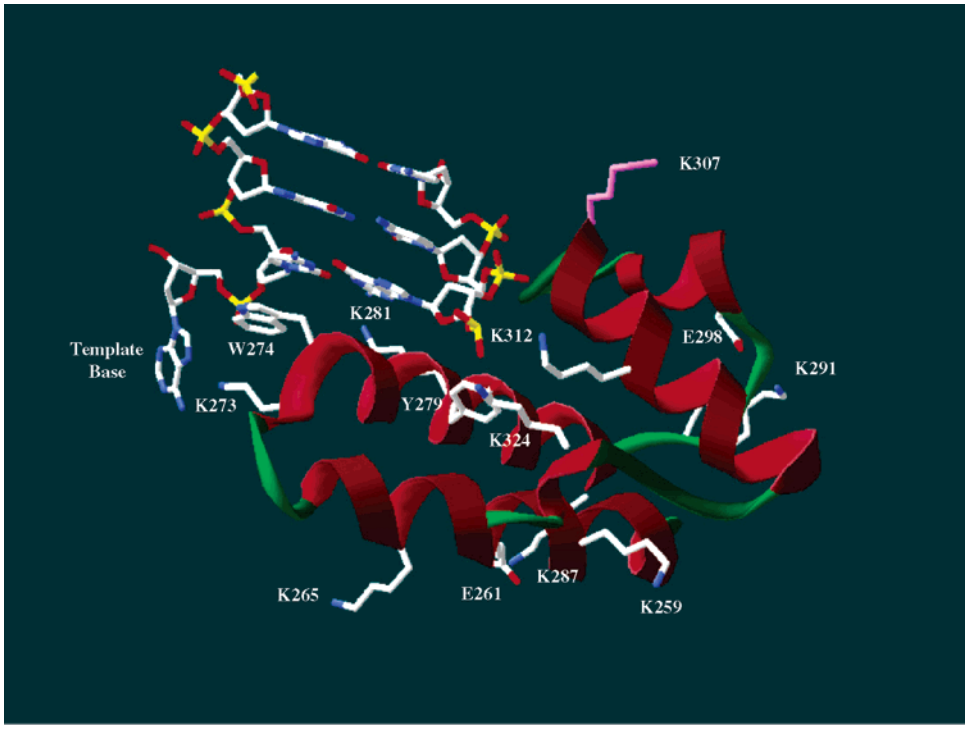

FIGURE 6: Crystal structure of the lyase domain of DNA Pol λ complexed with DNA. The structure includes some of the nucleotides that interact with the lyase domain, and most of the lysine side chains, as well as interacting residues E261, W274, Y279, and E298. K307 is shown in pink, since the position of this side chain was not determined in the X-ray crystal structure.

dimension, and the remaining residues do not experience significant shifts. On the basis of the structure of the Pol λ complex with a two-nucleotide gapped DNA substrate (PDB code: 1RZT) (*17*), the large shift perturbation observed for K273 probably results from an indirect effect of the DNA on the relative positions of K273 and W274 (Figure 6). Specifically, in the solution structure of Pol λ, the position of the W274 side chain is poorly defined, reflecting an absence of NOE restraints (*14*), while in the crystal structure corresponding to the complex with gapped DNA (PDB code: 1RZT) (*17*), W274 is positioned more rigidly due to the stacking interaction with the +1 template base (i.e., one nucleotide downstream of the coding template base and corresponding to C10 in the model DNA hairpin). A similar stacking interaction is observed with H34 and the +1 template base in Pol β (*18*). This positioning will reduce the averaging effect of the shift contribution from the disordered tryptophan and should result in an upfield ^{1}H shift due to the ring current, consistent with the observed K273 shift perturbation. Although the shift perturbations of K281, K307, and K312 are consistent with their location near the bound DNA, no significant shift would be predicted for K291 which is located on the opposite side of the protein. The sensitivity of the K291 shift to DNA binding may result from an indirect conformational change that perturbs the K291−E298 salt bridge or from nonspecific binding, although the latter interpretation is not supported by the absence of significant shifts for other remote lysine residues. In our experience, extreme chemical shift values that differ very significantly from those for the majority of residues of the same type tend to be highly sensitive to small structural perturbations.

Additions of DNA beyond the 1:1.3 molar ratio did not result in further chemical shifts, indicating that, at this ratio, the protein is nearly fully complexed with the DNA. On the basis of the 0.15 mM concentration of the protein used in these studies and the fact that at intermediate degree of complexation the resonances are in fast exchange between free and complexed values, we estimate the protein−hairpin DNA dissociation constant to be ~1 μM. This value is similar to the K_m values determined by kinetic measurements of the lyase activity of Pol β and its isolated lyase domain (*12*).

Formation of the Pol λ lyase domain−DNA complex resulted in some improvement in high pH stability of the protein, so that a useful spectrum could be obtained at a final titration pH value of 10.84. A general increase in the lysine pK_a values, with the average value increasing by 0.55 pH unit to 10.71 (Table 1), was observed for the protein−DNA complex. The fitted value for K312 increases by 1 pH unit to 10.58. In the 1RZT crystal structure of the complex of Pol λ (lacking the BRCT domain) with gapped DNA (Figure 6), the ϵ-amino group of K312 is 3.55 Å from the phosphorus atom in the 5′-phosphate group. This salt bridge interaction would be predicted to increase the pK_a of K312. In the complex with the DNA hairpin, the corresponding interaction would involve the 3′-phosphate group of the 5′-nucleotide; however, a similar interaction would be predicted. The observed increased pK_a value for K312 in the DNA complex is clearly not optimal for Schiff base formation. Similar results are obtained using the four-parameter fit, which yields a pK_a value of 10.50 for K312 (Table 2). The Hill coefficients obtained for the complex are consistently higher than those measured for the isolated domain, and nearly all values exceed 1.0. Although the basis for this is not completely clear, one effect of the DNA binding may be to shield many of the lysine residues from the effects of titration of the remote residues, reducing the negative cooperativity observed for the uncomplexed domain (Hill coefficients <1).

A plot of $\delta(^1H)$ vs $\delta(^{13}C)$ for K273 was of particular interest, since it differs dramatically from that obtained for

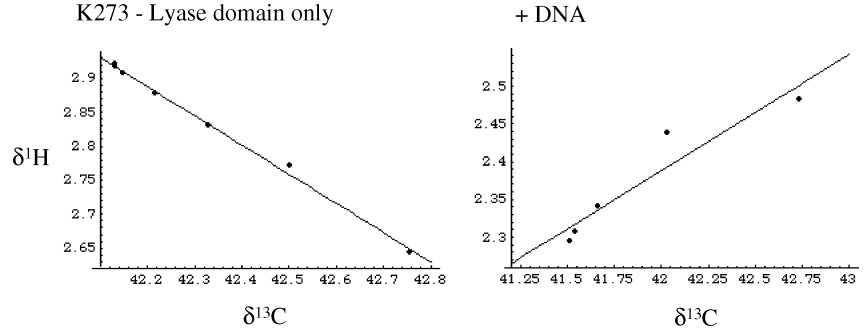

FIGURE 7: Plots of $\delta(^1H)$ vs $\delta(^{13}C)$ for residue K273: (left) isolated lyase domain; (right) lyase domain plus hairpin DNA.

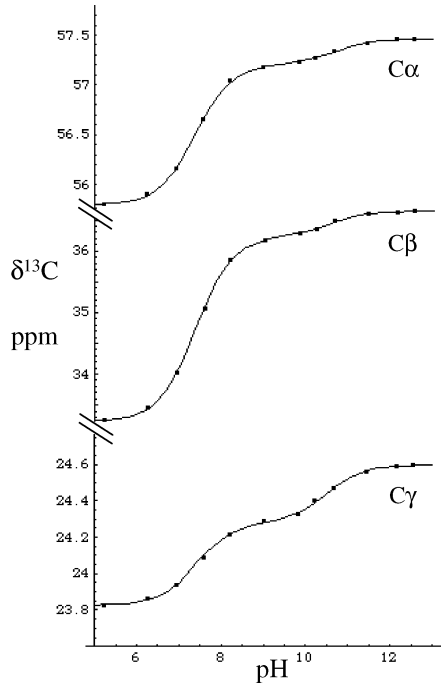

FIGURE 8: ^{13}C shifts for the C_α, C_β, and C_γ carbons of the K1 residue in the tripeptide KWK as a function of pH.

the uncomplexed lyase domain (Figure 7). Apparently, titration of K273 significantly perturbs the interaction with W274, leading to a markedly nonlinear plot. Potentially, there might be limited dissociation of the complex at higher pH, although the arginine residues involved in complex formation will not significantly deprotonate at pH 11. More probably, a localized, pH-dependent structural change contributes to the observed shift behavior.

pK_a Values of Lys-Trp-Lys, a Tripeptide with Lyase Activity. It has previously been demonstrated that peptides containing an aromatic residue flanked by basic residues possess significant lyase activity (*32*−*34*). Recently, Kurtz et al. (*32*) demonstrated that most of the apurinic/apyrimidinic (AP) lyase activity exhibited by the tripeptide Lys-Trp-Lys apparently results from an interaction with the N-terminal α-amino group rather than the lysyl ϵ-amino groups. A series of $^1H-^{13}C$ HSQC studies performed on the tripeptide as a function of pH yielded the expected pH-dependent shift behavior. Of the protonated carbons that were observed, C_α, C_β, and C_γ of Lys1 show the clearest dependence on titration of the N-terminal amino group (Figure 8). Fitting the data using eq 2 yielded pK_a values of 7.40 for the α-amino group, 10.51 for the ϵ-amino of Lys1, and 10.60 for the ϵ-amino of Lys3. These results demonstrate the importance of the lowered pK_a for activity in the model tripeptide system and are generally consistent with the pK_a estimates given by Kurtz et al. (*32*).

DISCUSSION

The N-terminal domain of DNA polymerase β and the corresponding central, 8 kDa domain of Pol λ have been reported to catalyze the loss of abasic deoxyribose-5′-phosphate (*5*, *9*). This is an essential and generally rate-limiting step in the DNA repair process (*1*−*4*). It has been demonstrated that the reaction proceeds via a β-elimination mechanism involving formation of a Schiff base intermediate (*9*, *13*). This type of chemistry is ubiquitous in biological systems. Matsumoto and Kim noted that β-elimination at AP sites is also catalyzed by basic proteins such as histones and polyamines (*9*), and they find that, in comparison with Pol β, similar activities require 10^3-fold more histone H1 or 10^6-fold higher spermine levels. Similar chemistry is also involved in nonenzymatic glycosylation reactions which are thought to contribute significantly to diabetic complications (*35*).

In general, formation of the Schiff base intermediate is viewed as resulting from a nucleophilic attack of the (unprotonated) amine on the aldehyde C-1′ of the open-chain sugar (*8*, *21*). Potentially, the reaction might also proceed as a substitution reaction with the furanose form of the deoxyribose in which the amino group of the reactive lysine replaces either the lactam O-4 ligand to C-1 or the C-1 hydroxyl group, analogous to proposals for the T4-pyrimidine dimer DNA glycosylase in which the base is displaced (*36*). For the substitution reaction, the leaving group (e.g. the C-1′ OH) becomes protonated so that the initial step in the reaction becomes a proton transfer from the (protonated) amino group. In either case it is beneficial to have an amino group with a pK_a value close to neutrality. This will either facilitate the proton-transfer step for the substitution reaction or allow formation of the nucleophilic (unprotonated) amine if the protonation step involves some other residue.

Consistent with this analysis, lysine residues involved in Schiff base chemistry typically exhibit significantly lowered pK_a values (*22*−*24*). In some cases, the need for a lower pK_a value is satisfied by utilizing the N-terminal amino group of the protein, e.g. T4 endonuclease V (*37*) and Fpg protein, an enzyme with DNA glycosyase/AP lyase activity (*38*, *39*). Kurtz et al. (*32*) have demonstrated that the lyase activity of the extensively studied KWK tripeptide (*32*−*34*) involves primarily a reaction with the N-terminal amino group, shown here to have a pK_a value of 7.40, rather than the ϵ-amino groups, shown here to have pK_a values of 10.51 and 10.60 (Figure 8).

The pK_a of 9.58 obtained for K312, while somewhat lower than the pK_a values for the remaining lysine residues, is substantially greater than optimal for Schiff base chemistry at physiological pH. One reason for the lowered pK_a value is presumably the high concentration of basic residues—R275, R308, R323, and K324, positioned near K312—as well as the high pI = 9.52 for the isolated domain. Thus, the average pK_a value determined for the 10 lysine sidehcains in the lyase domain was 10.16 (Table 1), significantly below the value of 10.80 obtained for the model compound N_α-acetyl lysine, which is typical of the values for lysine-containing model peptides in the literature (*29*). It is well established that an elevation of the protein pI is correlated with a depression in pK_a values (*40*). Formation of the protein−DNA complex effectively makes the protein more acidic, lowering the effective pI and hence increasing the pK_a values. Analogous but opposite effects are apparent in calbindin D_{9k}, for which a low pI value of 4.53 is associated with a high average lysine pK_a value of 12.07, while the addition of positively charged Ca^{2+} lowers the average pK_a value (*41*). Despite the relatively low pK_a for K312, the value is still more than 2 pH units above typical physiological pH values. Further, higher pK_a values are obtained from the Hill plot analysis of the uncomplexed domain (10.32) and from the titration of the lyase domain−hairpin DNA complex (10.58/10.50). The possibility of a significant error in the analysis of the K312 titration data due to titration of the nearby Y279 residue is unlikely, because the $\delta(^1H)/\delta(^{13}C)$ plot is well behaved and exhibits a slope similar to that for free N_α-acetyl lysine (Figure 4, Table 1). Hence, we conclude that the pK_a value for K312 does not appear to be optimized for Schiff base chemistry.

The conclusions of the pH titration study are completely consistent with recent structural data obtained for Pol β in complexes with gapped DNA substrates having stable, reduced acyclic, or cyclic (tetrahydrofuran) deoxyribose analogues at the 5′-terminus of the gap (*20*). These structures have several important implications for the lyase mechanism of Pol β and, presumably, Pol λ as well. (1) The deoxyribose-5′-phosphate analogue at the 5′-terminus of the downstream nucleotide is very distant from the entire polymerase domain ((*20*); also, see the Supporting Information). Consequently, there is no apparent basis for a contribution of the polymerase domain to the dRP lyase activity, other than an indirect effect resulting from enhanced affinity for the DNA substrate. (2) The structure of the complex is fairly open, allowing the deoxyribose analogue at the 5′ terminus to move away from the putative nucleophile. Thus, the C-1′ carbon on the tetrahydrofuran analogue is positioned 8.7 Å from the ϵ-amino nitrogen of K72 and the C-1′ carbon of the open-chain analogue is 10.1 Å from the K72 ϵ-amino nitrogen. These distances are significantly longer than those reported for mechanistically related DNA glycosylases. For example, in the complex formed from Fpg with a tetrahydrofuran-containing analogue, the distance between the tetrahydrofuran C-1′ and the proline Nα nucleophile is 4.3 Å (pdb code 1PM5) (*42*), and in a complex of the DNA glycosylase EndoIII with DNA containing an abasic site, the ϵ-amino group of K121 in the active site is 3.35 Å from the C-1′ of the deoxyribose (pdb code 1P59) (*31*).

The open structures obtained for the Pol β complexes with the abasic site analogues (*20*) are consistent with the relatively high pK value obtained for K312 of Pol λ in the present study. Typically, lysine residues exhibiting significantly perturbed pK values are buried in the interior of the protein. On the basis of the structures of the nonreactive complexes that were obtained, Prasad et al. concluded that although it is possible that the pK_a of K72 is shifted toward neutrality when it attacks at C1′, there is no obvious explanation for such a shift from the crystal structures. In the case of the Pol β complex discussed above, the catalytic activity of the enzyme was rationalized by a proposed rotation of the abasic deoxyribose-5′-phosphate group by ~120° about the 3′-phosphate group, which would swing the C-1′ of the abasic sugar into position to react with K72 (K312, in the case of Pol λ) (*20*).

The additional structural and pK data available suggested a reexamination of the kinetic data for the lyase reaction. Matsumoto and Kim (*9*) have reported that the lyase activities of the 8 kD domain and the full Pol β enzyme are comparable, while steady-state kinetic studies of Prasad et al. yielded k_{cat}/K_m values that were 7-fold lower for the 8 kD lyase domain than for the intact enzyme (*12*). On the basis of the effect of substrate concentration, it appears that the difference between the dRP lyase activity of Pol β and its isolated lyase domain can be completely attributed to a difference in the Michaelis constant, since k_{cat} is actually slightly higher for the 8 kD domain. Prasad et al. also studied the pH dependence of the dRP lyase reaction by both intact Pol β and by the isolated 8 kD lyase domain (*12*). The low sensitivity of the catalytic efficiency toward pH and the overall lower catalytic efficiency of the 8 kDa domain (Table 1) were interpreted to be consistent with a critical amine residue being protonated over the entire pH range (*12*). However, a fit of the data in Figure 7 of this reference indicates that the activity data for both the full enzyme and the isolated lyase domain are well described by a single titratable group with a pK of 6.4 for the intact enzyme or 6.9 for the 8 kD domain (Supporting Information). Although this pK could conceivably correspond to a lysine residue in an environment that depresses the pK value, it also might correspond to a another titrable residue. For example, Feng et al. have proposed that the conserved Glu71 residue, corresponding to Glu311 in Pol λ, facilitates the hydrogen abstraction reaction of the Schiff base intermediate (*8*). Hence, this represents one alternative candidate for the titrable residue implicated by the pH-dependent activity studies. Further, it is difficult to reconcile the relatively small magnitude of the pH-dependent changes observed with protonation of the critical lysine nucleophile.

The titration behavior of the isolated Pol λ lyase domain is thus seen to fit well with the much more extensive biochemical and structural data that have been obtained over the past decade for Pol β. The structural alignment obtained previously shows that all of the critical residues proposed to be involved in the β-elimination reaction of Pol β are present in Pol λ. However, several of the residue substitutions that occur in going from Pol β to Pol λ have interesting mechanistic implications. In particular, protonation of O4′ as a catalytic step in the opening of the deoxyribose ring was proposed to involve Lys35 or Lys60 in Pol β (*8*). Since both residues correspond to arginine in the Pol λ structure (*14*), an alternative mechanism must be sought. The most likely explanations are either that the enzyme works only

on the 1% of the deoxyribose that is in the open-chain, aldehyde form (*43*) or that it K312 itself serves as the proton donor, simultaneously creating the nucleophilic, unprotonated form for attack at C-1′ (see Figure 3 in (*44*)).

Given that nonenzymatic glycosylation occurs in the absence of a specific binding interaction, it is perhaps not surprising that the Schiff base chemistry can be dramatically accelerated by a binding interaction that positions the lysine side chain near the reactive abasic site, even in the absence of a significant lowering of the lysine p*K* value. Since the lyase reaction appears to be a rate-limiting step in the repair process under many conditions (*1*−*4*), it is somewhat surprising that the pK_a of the reactive residue is not better optimized for the required chemical reaction. One possible interpretation of this result is suggested by the recent observation of Wong & Demple (*45*) that the AP endonuclease Ape1 significantly accelerates the dRP lyase reaction of Pol β. Conceivably, such an interaction with Pol λ might alter the structure of the complex with the abasic site, changing the environment of K312 and hence its pK_a, or the Ape1 might supply another low-pK_a amino group for the reaction. Another interpretation of the failure of Pol λ/Pol β to optimize the dRP lyase reaction is suggested by studies of fibroblasts from Pol β-null mice, demonstrating that the presence of the dRP group serves as an important signal of DNA damage so that a function in signaling the status of DNA damage may supersede the need to rapidly effect the repair (*4*). Sobol et al. have demonstrated that persistence of the dRP moiety in DNA results in a hypersensitivity phenotype of Pol β null cells that may trigger downstream events such as apoptosis and necrotic cell death (*4*). The possibility that the dRP moiety is an important signal for DNA damage has received additional support from a report that the transcriptional coactivator P300 selectively acetylates K72 of Pol β (*46*), apparently supporting a regulatory role for the dRP lyase reaction in the DNA damage response process. These observations suggesting a role of the deoxyribose-5′-phosphate flap in signaling DNA damage may represent an important constraint on its removal from damaged DNA, so that the lyase domains of Pol β and Pol λ are not subject to a simple optimization of enzymatic chemistry which would result in a more optimal dRP lyase reaction.

Another consideration related to the apparent lack of optimization of the dRP lyase reaction may be the need for repair enzymes such as Pol β and Pol λ to deal with a more heterogeneous substrate population than is encountered by typical enzymes. In particular, it is known that these enzymes can fill gaps ranging from one to five nucleotides in length. There is at this point limited structural data on how these gaps of different lengths are dealt with, and it is conceivable that the position of the deoxyribose-5′-phosphate located immediately after the gap may vary somewhat as Pol λ forms complexes with these variable substrate populations. In this case, it may have proven more efficacious to utilize the flexibility of a surface lysine residue than to bury the entire 5′-terminus in an enzyme pocket requiring an exact fit. Thus, the characteristics of the active site of the lyase domain may represent a compromise of flexibility with catalytic efficiency.

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SUPPORTING INFORMATION AVAILABLE

Supplementary material includes additional structures of Poly λ-DNA complexes, a replot of the pH-dependent relative activity data originally obtained by Prasad et al. (*12*), and additional plots of the δ^1H vs δ^{13}C data for K287 and K324 obtained after dropping the three low pH data points. This material is available free of charge via the Internet at http://pubs.acs.org.

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