Pergamon

Tetrahedron, Vol. 53, No. 35, pp. 12057-12066, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00768-0

Insights into the Mechanism of the β-Elimination Catalyzed by the N-terminal Domain of DNA Polymerase β

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Abstract: The N-terminal domain of DNA polymerase β carries an activity for excision of deoxyribose 5-phosphate from DNA at an interaction interface that includes a helix-hairpin-helix motif containing Lys-68 and Lys-72 and an adjacent Ω loop containing His-34 and Lys-35. His-34 displays a low pK_a (5.7) due to proximity to Lys-35. In a proposed mechanism, Lys-68 protonates the hemiacetal O4' and His-34 stabilizes the protonated aldehyde-type species, possibly accepting the proton. In one of two possible mechanisms, Lys-68 or Lys-72 forms a Schiff's base with the substrate. His-34 can function in C2' deprotonation for the Lys-68 Schiff's base intermediate, while the proton would be transferred to water for the Lys-72 Schiff's base. Lys-35 stabilizes the phosphomonoester leaving group in either case.

INTRODUCTION

DNA polymerase β (β-Pol) consists of an independently folded N-terminal domain of 87 amino acid residues and a folded C-terminal domain of 247 amino acid residues.[1,2] The C-terminal domain carries template-dependent nucleotidyl transferase activity.[3] The N-terminal domain was originally characterized as a ssDNA template binding domain and later found to have DNA gap binding function.[1,4] More recently, the N-terminal domain was found to catalyze removal of 5'-terminal deoxyribose 5'-phosphate from abasic site duplex DNA.[5]

The N-terminal domain consists of four helices. Helix 1 and 2 are antiparallel and cross the antiparallel helix-3-turn-helix-4, which forms a helix-hairpin-helix (HhH) motif.[6-8] The HhH motif is structurally identical to the HhH motif in endonuclease III[9] and in the 3-MeA DNA glycosylase.[10] Sequence conservation of the HhH motif is found in a large number of DNA repair enzymes that share no other homology.[11,12] The interaction interface for ssDNA and dsDNA binding to the β-Pol N-terminal domain includes the HhH motif containing Lys-68 and Lys-72 and an adjacent Ω loop containing His-34 and Lys-35.[6,7]

The β-Pol N-terminal domain and a Lys-72 to Ala mutant of the N-terminal domain were previously shown to form a Schiff's base intermediate in excision of deoxyribose 5'-phosphate from duplex DNA.[13] Endonuclease III and the β-Pol N-terminal domain are non-homologous, but each have the HhH motif in common with a conserved lysine occupying position 13 of the motif in each enzyme.[8] Like the β-Pol N-terminal domain, endonuclease III forms a Schiff's base intermediate.[14] Mutation of the structurally conserved lysine in endonuclease III (corresponding to Lys-68 in the β-Pol N-terminal domain) results in a 10^5-fold decrease in AP lyase activity.[9] Endonuclease III has been shown to interact primarily with the minor groove near an abasic site in DNA.[15] Based on these combined findings, a model of abasic site DNA interaction by the HhH in the β-Pol N-terminal domain and in endonuclease III was recently proposed.[8]

The modeling of an abasic site DNA duplex in complex with the β-Pol N-terminal domain was used to address the mechanism of lesion detection by the HhH motif in DNA repair enzymes.[8] The model provided an explanation for reactivity by a lysine-HhH at damaged nucleotides and non-reactivity by the lysine-HhH at non-damaged nucleotides. For the β-Pol N-terminal domain and endonuclease III, the superimposed lysine-HhH in each enzyme made contacts in the minor groove. For the β-Pol N-terminal domain, His-34 and Lys-35 interacted in the major groove with the imidazole ring of His-34 fitting into the empty base position in the DNA. In the lesion detection mechanism, the lysine-HhH in DNA repair enzymes is rendered non-nucleophilic through interaction with either the O2 or N3 H-bond acceptors along the base stack in the minor groove. A missing H-bond acceptor, due either to an abasic site or a damaged and improperly positioned base, results in transfer of the proton from the lysine-HhH to the substrate and cleavage of the of the O4'-C1' bond of the hemiacetal or

damaged nucleotide. In this report, catalytic roles of amino acid residues involved in deoxyribose 5'-phosphate excision by the β-Pol N-terminal domain are proposed.

EXPERIMENTAL

Materials. The cloned β-Pol N-terminal domain of sequence ^{2}SKRKAPQE-^{10}TLNGGITDML-^{20}VELANFEKNV-^{30}SQAIHKYNAY-^{40}RKAASVIAKY-^{50}PHKIKSGAEA-^{60}KKLPGVGTKI-^{70}AEKIDEFLAT-^{80}GKLRKLEK was overproduced and purified as described previously.[6] The ^{15}N-labeled protein was similarly prepared. The purified protein was exchanged into 100 mM NaCl containing 5 mM Tris-d_{11} by Sephadex G15 gel filtration chromatography as described previously.[6]

NMR Sample Conditions. The NMR sample for TOCSY contained approximately 1 mM β-Pol N-terminal domain in 99.9% D_2O containing 100 mM NaCl. The samples were concentrated and/or exchanged into D_2O using a centricon-3 (Amicon) centrifugal ultrafiltration device. For modification of the N-terminal with pyridoxal phosphate, the pH was 6.8. For collection of ^{1}H-^{15}N HSQC spectra, the ^{15}N-labeled β-Pol N-terminal domain was at pH 6.8 in 90% H_2O/10% D_2O containing 100 mM NaCl . The temperature for data collection was 27° C.

NMR Methods. Two-dimensional total correlation spectroscopy (TOCSY) experiments[16] were performed on a Varian Unity-plus 500 MHz NMR spectrometer. The pulse sequence utilized a MLEV-17 spin-lock for 70 ms and States-TPPI phase cycling for collection of phase sensitive data in the t_1 dimension. The spectra were recorded using 16 scans per t_1 increment. The ^{1}H-^{15}N HSQC spectrum for the pyridoxal phosphate modified N-terminal domain was collected using a sensitivity enhanced pulse sequence with gradient coherence selection and gradient water suppression as published previously.[17] The NMR data were processed and analyzed using either the programs PROSA and XEASY[18] or Felix95 (BioSym Technologies).

β-Pol N-terminal Domain-pH Titration and Data Analysis. The pH titration was performed by first increasing the pH to 9.9 followed by stepwise addition of 100 mM HCl and recording of two-dimensional TOCSY data. The chemical shifts of the histidine Hε2 and Hδ1 protons for the Hε2-Hδ1 cross peak in the TOCSY spectrum were plotted as a function of pH after fitting by a linear least squares to equation I as described previously.[19]

$$\delta_{(pH)} = \frac{\delta_{HB} + \delta_B 10^{pH-pKa}}{1 + 10^{pH-pKa}} \qquad (I)$$

HPLC. Reverse phase HPLC was performed on a Rainin HPLC system employing a C18 column (10 × 250 mm) and a linear gradient of acetonitrile and water.

Mass Spectrometry. Mass spectrometry was performed on a Kratos Kompact MALDI IV. The ^{15}N-labeled β-Pol N-terminal domain was prepared for mass spectrometry by HPLC elution from a reverse phase C18 column or by direct dilution of the NMR sample. For molecular mass characterization of the pyridoxal phosphate modified N-terminal domain, pyridoxal phosphate was added in a 10-fold excess (10X) over the N-terminal domain at pH 6.8 and was allowed to react to completion for a period of greater than 2 h.

RESULTS AND DISCUSSION

Two-dimensional TOCSY NMR spectra of the β-Pol N-terminal domain were used to characterize the pH dependence of the proton chemical shifts of histidine, tyrosine, and phenylalanine aromatic resonances (Figure 1). The typical pK_a of a histidine in a random coil peptide is 6.5-6.7. His-34 displays a reduced pK_a of 5.7 indicating that this side chain will be greater than 90% deprotonated at neutral pH. Analysis of the 55 superimposed NMR solution structures shows that the reduced pK_a for His-34 results from the neighboring protonated side chain of Lys-35 (Figure 2). The typical pK_a for a non-interacting lysine at the surface of a protein is ~10. Assuming conservation in free energy, Lys-35 would be predicted to display a corresponding increase in its pK_a

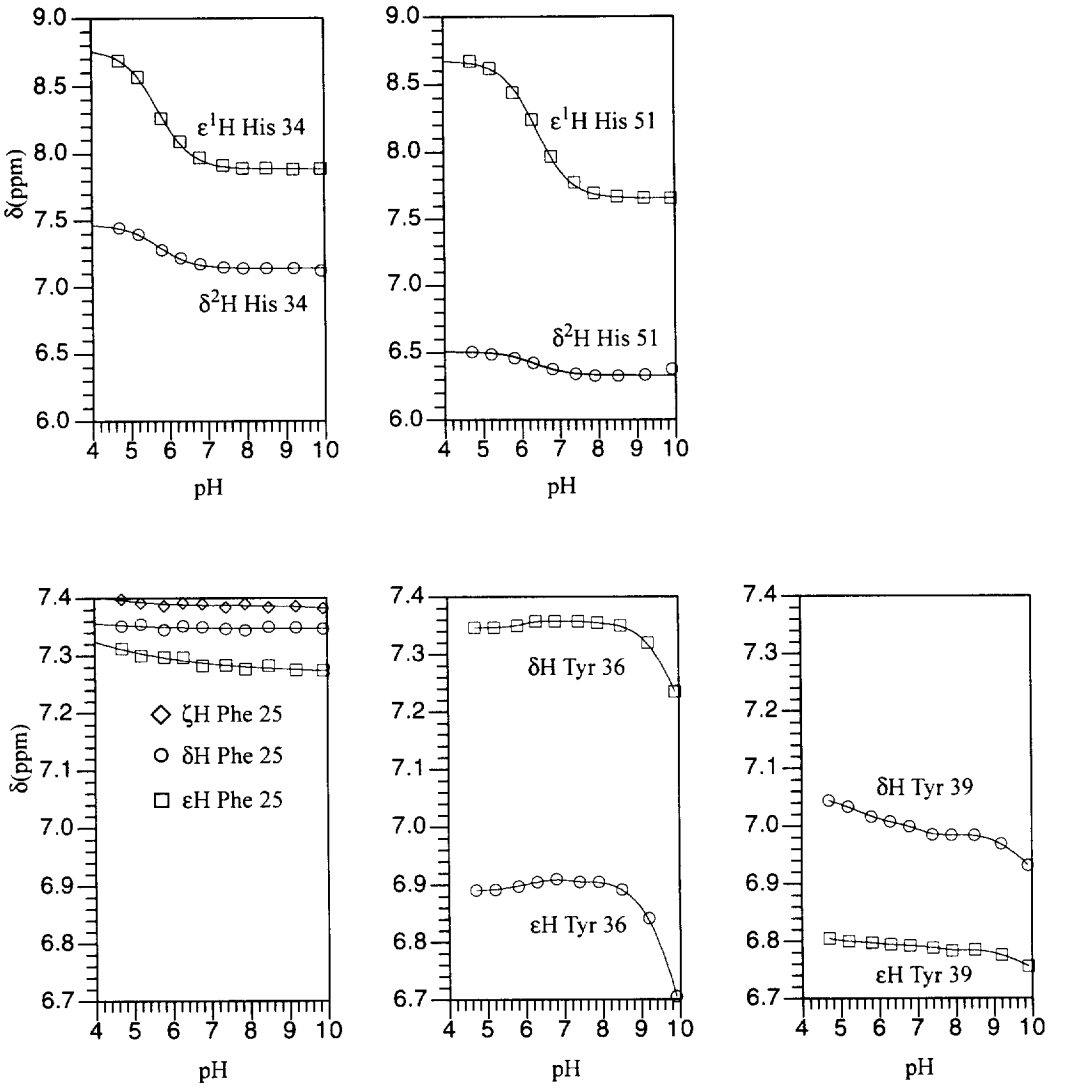


Figure 1. NMR pH titration curves for His-34 (pK_a = 5.7) and His-51 (pK_a = 6.4) and the indirect effect of His-34 protonation on Tyr-39 and Phe-25 aromatic chemical shifts. The Tyr-36 and Tyr-39 hydroxyls show partial deprotonation between pH 8 and 10 consistent with typical pK_a values of ~10. The chemical shifts for the designated proton resonances were measured from two-dimensional TOCSY spectra at each pH.

to ~11. Such an increased pK_a would make Lys-35 an unlikely candidate for Schiff's base formation, since only ~0.01% would be unprotonated at neutral pH. Protonation of His-34 induces shifts of ~0.05 ppm in the aromatic δH protons of Y39, which are buried between the lysine side chains of Lys-35 and Lys-72 indicating positive charge effects are transferred to this region of the DNA interaction surface. Similarly, effects of ~0.04 ppm were observed on the ξH proton of Phe-25, which is at the fringe of the interaction interface. A much smaller effect (<0.01 ppm) was observed on Tyr-36 which is partially exposed on the opposite face of the domain.

Using NMR spectroscopy, we have examined the possibility that pyridoxal phosphate could preferentially react with a low pK_a lysine in the β-Pol N-terminal domain. A two-dimensional ^1H-^{15}N fingerprint spectrum of the backbone amides in the N-terminal domain was recorded before and after addition of 1 and 2 equivalents of pyridoxal phosphate. For a single lysine modification, the most straightforward result would be discrete changes in the spectrum with the pattern for the un-modified protein changing to a new pattern of cross peaks of equal number and intensity. Changes might be expected to be localized near the modification site. The spectrum obtained at one equivalent of pyridoxal phosphate (Figure 3) showed doubling and tripling of cross peaks from amides not closely related in the three-dimensional structure. A large number of new cross peaks were observed. At 1X and 2X pyridoxal phosphate, the cross peaks for Lys-48, Lys-54, Lys-60, Lys-72, and to a lesser extent Lys-61, showed either dramatic loss of intensity and a corresponding gain in intensity in neighboring new cross peaks or peak doubling. The cross peak for Lys-68 was too weak for detection of a modification.

The ratios of the intensities of the doubled cross-peaks was an indication of more than one site of modification. At 1X pyridoxal phosphate, Lys-60 showed splitting into approximately equal intensity peaks (one corresponding to the modified and one corresponding to the un-modified species) (Figure 2B). At 2X pyridoxal phosphate, the modified species for Lys-60 increased to >50%. Lys-72 showed only a slight loss of cross peak intensity at 1X pyridoxal phosphate (much less than 50% loss) and a much larger loss of intensity at 2X pyridoxal phosphate (Figure 2C). Two weak cross peaks at proton chemical shifts slightly upfield of the unmodified Lys-72 cross peak grew in intensity at 2X pyridoxal phosphate. The results correspond to a second modification at Lys-72 different from the modification measured at Lys-60, and a third modification at a site that affects Lys-72. For Lys-48, a second minor modification peak and a slight doubling of the unmodified peak was observed at 1X pyridoxal phosphate that increased at 2X pyridoxal phosphate (Figure 2E). Lys-35 displayed a weak cross peak for the un-modified protein and at 2X pyridoxal phosphate suggesting little or no modification.

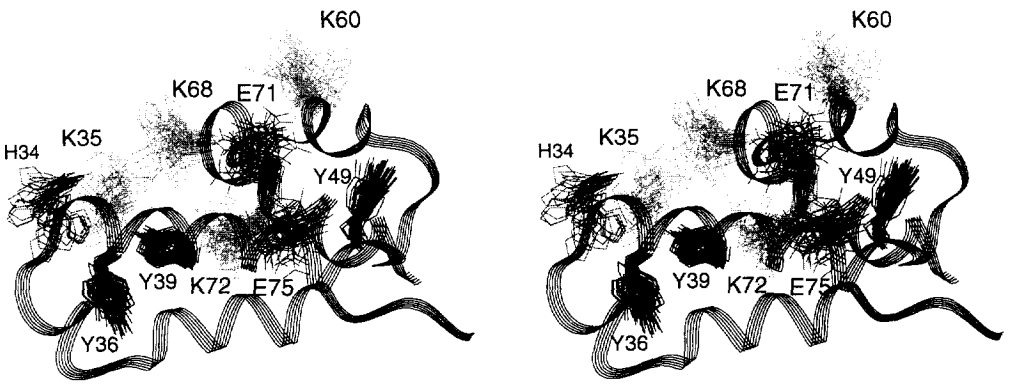


Figure 2. A convergent stereo ribbon representation of the NMR structure of the β-Pol N-terminal domain (minimized average structure, 1bno).[7] Shown are several of the flexible side chains (H34 and K35 in the Ω loop, K60 in helix-3, K68 in the flexible segment at the N-terminus of helix-4, and K72, E71, and E75 in helix-4). Also shown are the well-ordered tyrosine side chains (Y36, Y39, and Y49) in the 55 conformers (1bnp).[7]

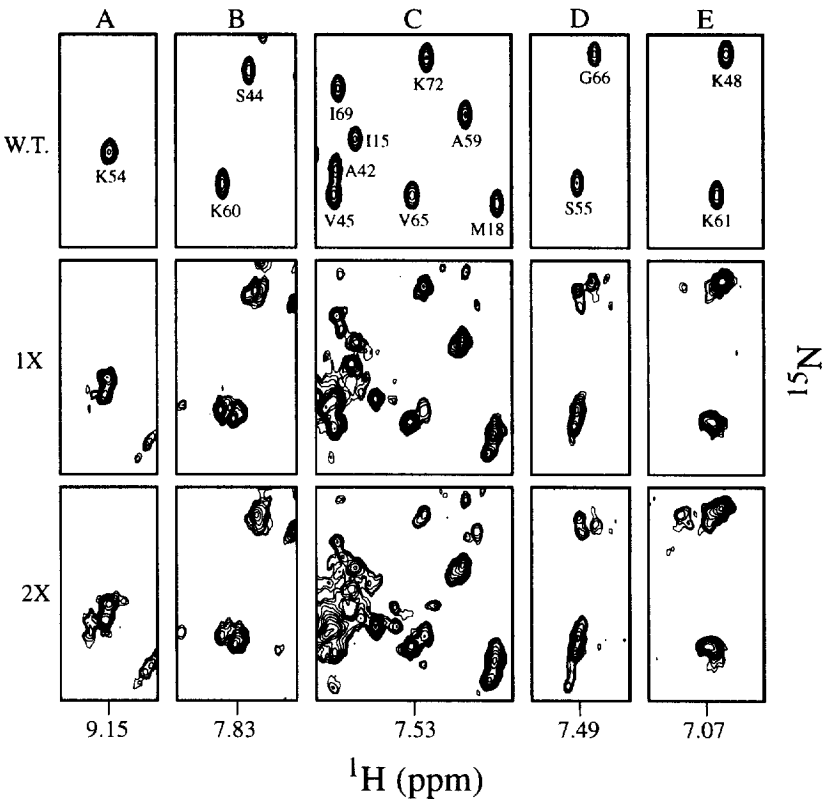


Figure 3. Selected regions (A-E) of the ^{1}H-^{15}N HSQC spectrum of the β-Pol N-terminal domain (W.T.), the N-terminal domain on addition of 1 equivalent of pyridoxal phosphate (1X), and on addition of 2 equivalents of pyridoxal phosphate (2X).

Pyridoxal phosphate was previously found to inhibit polymerase catalysis by β-Pol, and 4 moles of pyridoxal phosphate were found to be incorporated per mole of intact β-Pol.[20] Lys-72 was found to form a Schiff's base with pyridoxal phosphate by amino acid sequence analysis of tryptic peptides.[20] The Lys-72 modification was blocked by DNA template-primer-dNTP binding.[20] Lys-68 and Lys-81 were found un-modified, since trypsin proteolysis occurred at this residues.[20] The ^{1}H-^{15}N HSQC spectra indicate that more than one lysine was modified by the action of pyridoxal phosphate on the β-Pol N-terminal domain. The NMR spectra do not indicate whether the multiple modifications were on the same protein or different protein molecules. At 1X pyridoxal phosphate, no free pyridoxal phosphate was found by HPLC indicating a complete reaction.

After treatment with a 10-fold excess of pyridoxal phosphate, the β-Pol N-terminal domain was found to have molecular masses corresponding to 1 to 6 pyridoxal phosphate additions per protein molecule (Figure 4). The intensity of the peaks suggest that covalent modification by 2, 3, and 4 pyridoxal phosphates are most favored, even though, with 15 lysines in the β-Pol N-terminal domain, up to ten modifications per protein were possible. For each product containing one or more covalently attached pyridoxal phosphate molecules, a second product of lower mass was observed that differed in mass by 99 ± 5 amu. A species with a molecular mass difference of 99 amu was not observed for the un-modified protein. A mass difference of 95 amu is consistent with an elimination of a single inorganic phosphate.

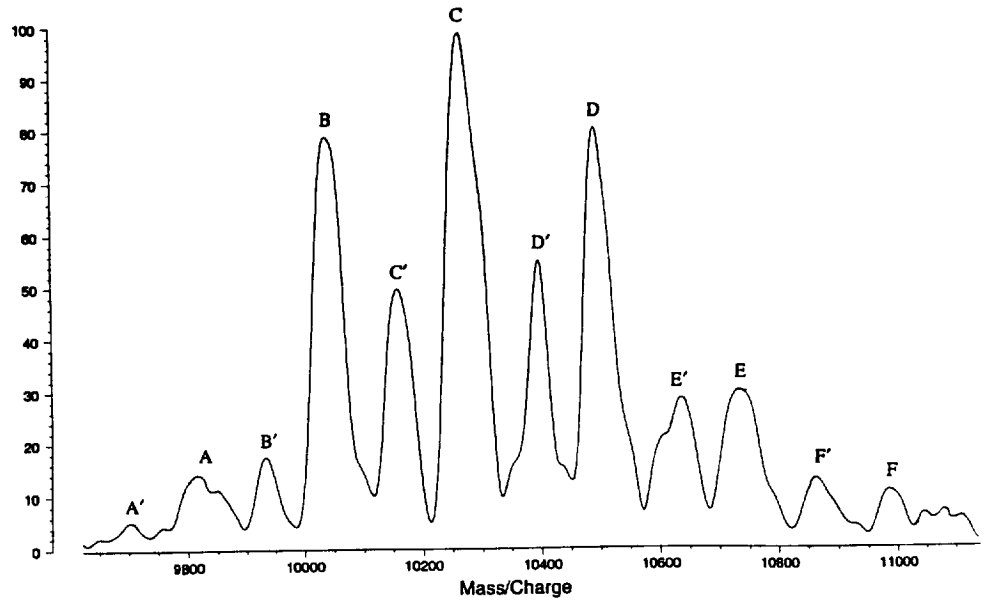
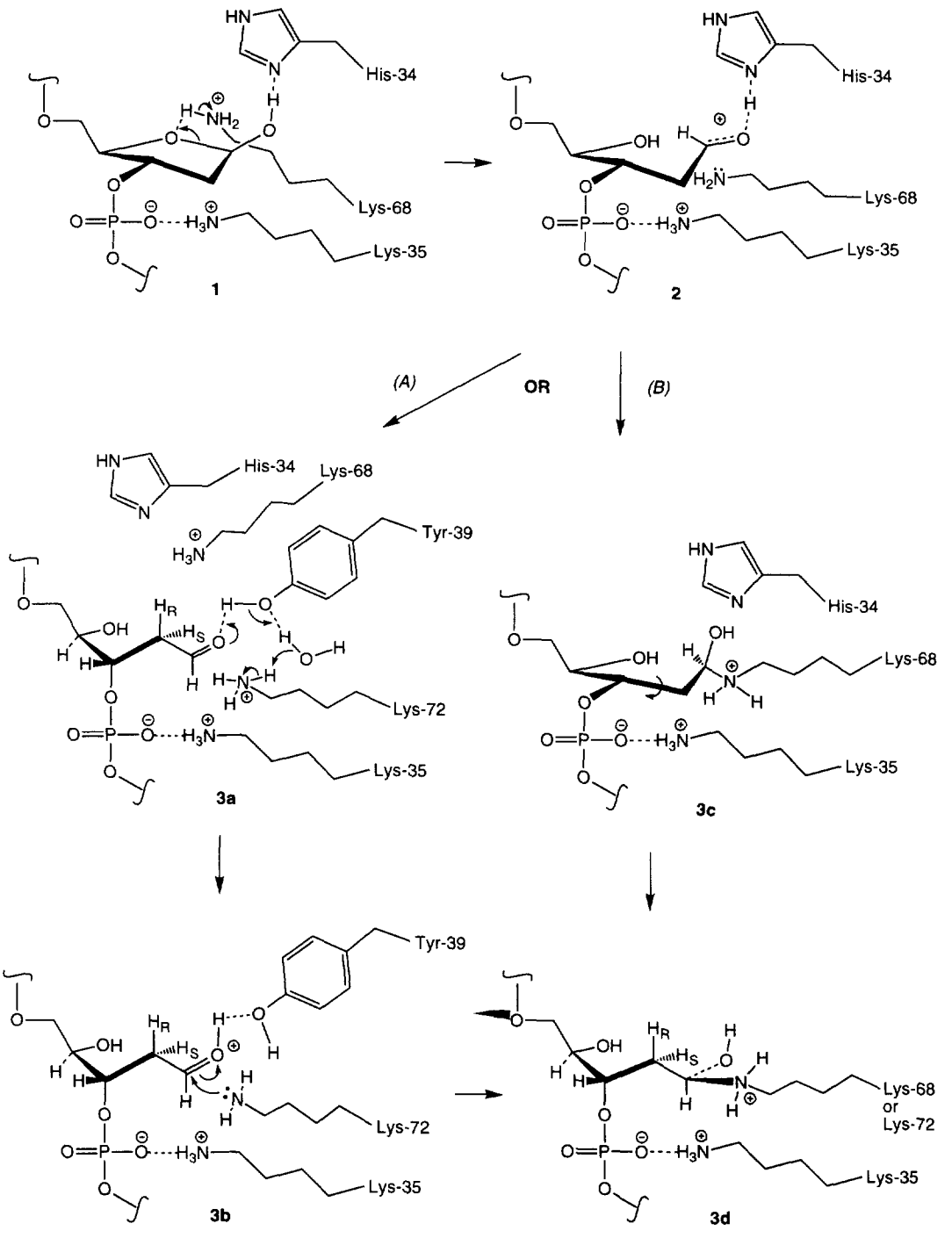


Figure 4. MALDI mass spectrometry analysis of the β-Pol N-terminal domain after modification with a 10-fold excess of pyridoxal phosphate (PLP). Molecular masses indicate the following number of covalent incorporations: peak A, 1 PLP; peak B, 2 PLP; peak C, 3 PLP; peak D, 4 PLP; peak E, 5 PLP, peak F, 6 PLP. Covalent incorporation of 2, 3, and 4 PLP form the major products. Peaks A'-F' correspond to a singular loss of a molecular mass of 99 ± 5 amu with respect to peaks A-F.

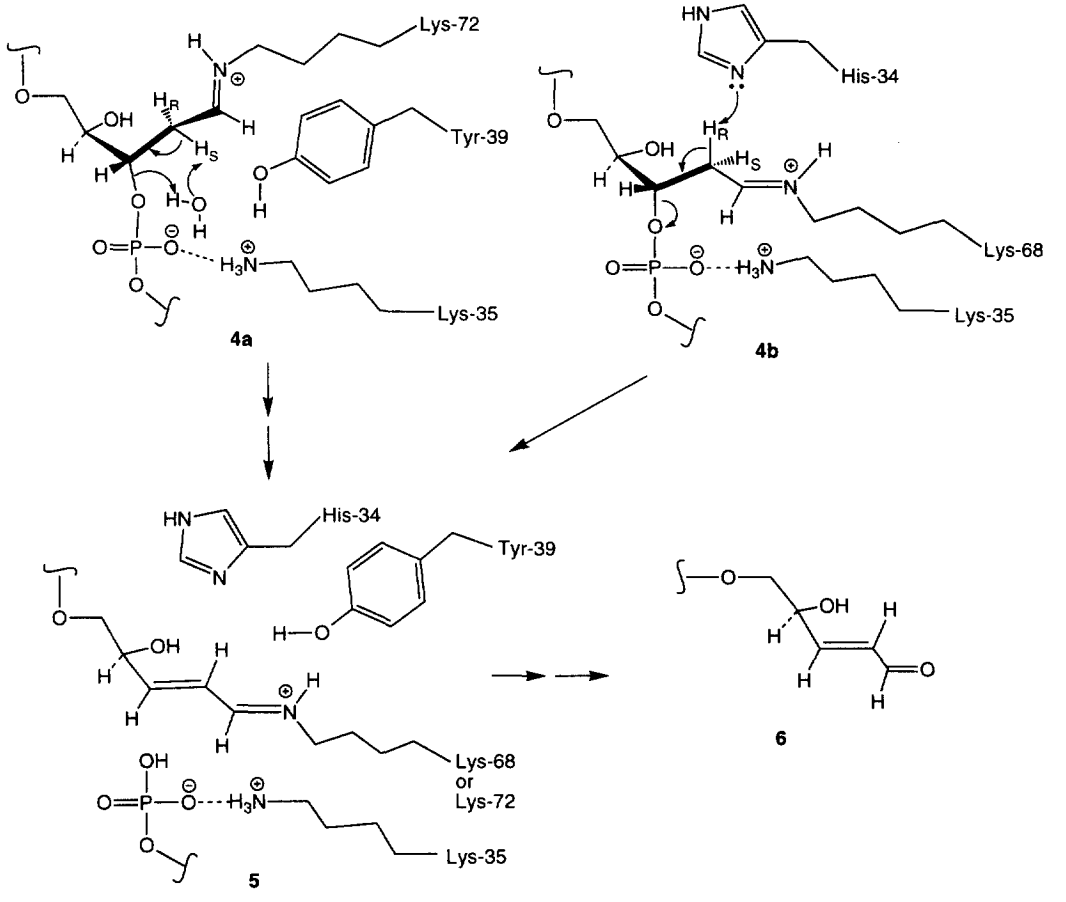
The strongest evidence implicating Lys-68 in Schiff's base formation is the finding that a mutation of the corresponding lysine in the HhH of endonuclease III reduced the AP lyase activity for this enzyme by 10^5 fold.[9] The pyridoxal phosphate reactivity data that we and others[20] have found suggest that Schiff's base formation at the lysine-HhH could be due to formation of a specific active site complex. Lys-35 appears not to be a candidate for Schiff's base formation. For DNA containing deoxyribose 5'-phosphate, a Schiff's base intermediate has been trapped for the Lys-72 to Ala mutant of the N-terminal domain, albeit at reduced efficiency.[13] The loss in efficiency of Schiff's base formation for the Lys-72 to Ala mutant might suggest that the charge associated with Lys-72 contributes to the reactivity of the Lys-68 side chain. However, more recent Lys to Ala mutational data indicate that Lys-72 contributes to the deoxyribose phosphodiesterase β-elimination reaction while Lys-68 is dispensable. Although Lys-60 and Lys-61 in the HhH motif are candidates for Schiff's base formation, neither of these lysines are conserved among HhH repair enzymes. Since 2-4 lysines on the β-Pol N-terminal domain react with pyridoxal phosphate, Lys-68 or Lys-72 reactivity likely results from formation of a specific complex.

In the first step of the lyase catalyzed reaction, we propose that Lys-68 acts as a general acid. The O4'-C1' bond is broken in a bi-molecular electrophilic substitution (A-S_E2) of the ε-NH_3^+ proton of Lys-68 on the O4' oxygen of the hemiacetal **1** to produce a protonated aldehyde (or carbonium ion) intermediate **2**. His-34 can H-bond to the 1'-OH and may act as a general base by fully accepting the 1'-OH proton, thereby inducing O4'-C1' bond cleavage. The positioning of Lys-68 between Lys-34, Lys-41, Lys-60, and Lys-72 could provide a substantial electrostatic contribution to proton donation by Lys-68. We also note that as for the β-Pol N-terminal domain, the lysine-HhH in endonuclease III is surrounded by positive charge. The propensity for polyamines to catalyze elimination reactions, although at significantly lower rates, may similarly result from partial deprotonation at an amine as a result of surrounding positive charge. In one of two possible mechanisms, either the aldehyde form of the substrate changes conformation on the enzyme together with nucleophilic attack by Lys-72 as shown in **3a-b** via *(A)*, or there is a direct nucleophilic attack by the conserved Lys-68 to yield **3c** via *(B)*.


His-34
Lys-68
Lys-35
1
2
(A)
OR
(B)
Tyr-39
Lys-72
3a
3c
3b
3d
Lys-68
or
Lys-72

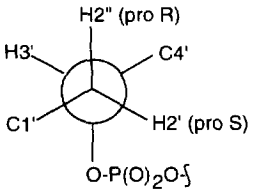
As shown in **3a**, Tyr-39 appears to be the only candidate, other than water, available for shuttling a proton to the aldehyde carbonyl oxygen. Nucleophilic attack via either mechanism leads to a carbinolamine **3d**, which would undergo dehydration to the Schiff's base **4a** or **4b**. The Schiff's base **4a** could undergo a *syn* elimination in a manner similar to that proposed for endonuclease III. The Schiff's base **4b** could undergo either *syn* or *anti* elimination. Modeling for Lys-68 Schiff's base formation suggests that His-34 could assist in C2' proton removal, while modeling for the Lys-72 Schiff's base suggests that proton removal can likely only be facilitated by water in conjunction with the leaving phosphomonoester. Proton abstraction by the His-34 side chain would likely proceed via an *anti* elimination on the basis of structural modeling of the complex and would require a considerable distortion of the intact phosphate backbone in order to accommodate the conformation **4b** leading to the *trans* geometry. The Schiff's base **5** would be hydrolyzed to the αβ-unsaturated product **6**.

An Asp-138 to Asn mutant of endonuclease III displayed a 100-fold reduced k_{cat} for the AP lyase catalyzed reaction.[9] Asp-138 in endonuclease III and His-34 in β-Pol may play similar roles, since they are positioned similarly on HhH superimposition. For endonuclease III, the pro S proton (H2') was abstracted from the C2' carbon to yield a *trans* product, indicating that the phosphomonoester group leaves from the C3' carbon in a *syn* configuration.[21] On the basis of this finding, Mazumder et al. suggested an intramolecular proton transfer from the C2' carbon to the phosphomonoester group in the β-elimination catalyzed by endonuclease III, as has been observed in enzymes that eliminate inorganic phosphate.[21] As pointed out by Mazumder et al., a phosphomono-



ester is considerably more basic than a phosphodiester, and thus elimination of phosphate via this mechanism will be considerably more facile than elimination of a phosphomonoester. The role of Lys-35 in the β-Pol N-terminal domain in stabilizing the leaving phosphomonoester group is supported by preliminary mutational data that will be presented elsewhere.

For completeness we consider the possibility of either *cis* or *trans* products in the β-elimination and the mechanism of water removal from the carbinolamine. *Trans* products require rotation about the C1'-C2' bond either before Schiff's base formation, as shown for the conversion of **2** to **3a**, or after formation of the carbinolamine as shown for the conversion of **3c** to **3d**. Newman projections show that rotamer **7** leads to a *trans* product, while rotamer **8** leads to a *cis* product. The *trans* configuration about C2'-C3' was found in the product from the AP lyase reaction catalyzed by endonuclease III.[21] Similarly, the *trans* product was found for the uncatalyzed β-elimination reaction at pH 13.[21] An *anti* elimination for rotamer **7** would proceed via abstraction of the pro R proton (H2"). A *syn* elimination for rotamer **7** would proceed via abstraction of the pro S proton (H2'). *Cis* products from rotamer **8** are less common in β-eliminations. Water removal from the carbinolamine would likely occur via a transition state **9** involving the interaction of an intervening water. Alternatively a hydroxyl group on the protein could serve this role.



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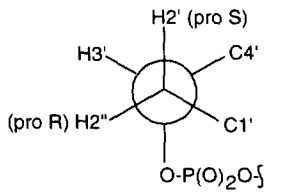

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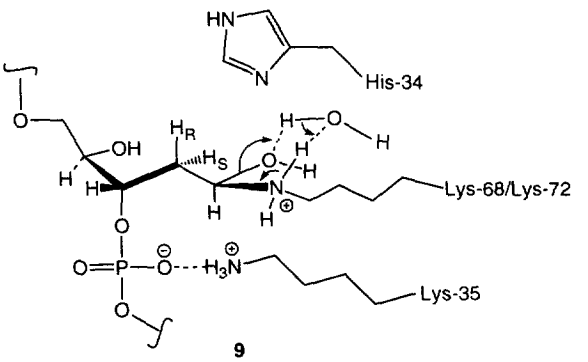

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We have discussed β-elimination mechanisms for abasic nucleotide cleavage catalyzed by the β-Pol N-terminal domain, that are applicable to AP sites containing a phosphomonoester at the 5'-carbon of the abasic site (preincised) or to an intact AP site containing a phosphodiester at the 5' carbon. Products for the preincised abasic site would be the 4-hydroxy-2,3-pentenal 5-phosphate **6**, whereas the product for the intact abasic site would be the 4-hydroxy-2,3-pentenal 5-phosphodiester. Recent evidence suggests that the N-terminal domain catalyzes excision at intact abasic sites but at a significantly reduced rate in comparison to the β-elimination at precleaved sites (Prasad & Wilson, unpublished). The differences in catalysis are likely correlated with the inability of the intact substrate to readily form a Schiff's base intermediate. Rotation about the phosphodiester bond to be cleaved would be facile in the preincised substrate in the aldehydic form and would allow the substrate to form a Schiff's base intermediate with either Lys-68 or Lys-72.

While the mechanisms are speculative, presently constructed mutants of the β-Pol N-terminal domain at residues that include His-34, Lys-35, Lys-68, and Lys-72 offer a means of exploring each of the predictions. Careful analysis of the effects of the mutation on both k_{cat} and K_M for the β-elimination reaction will be necessary. The possibility that the Lys-68 to Ala mutant or the Lys-72 to Ala mutant presently being explored may catalyze a residual reaction via a switched mechanism will also need to be considered. The reactivity of lysines toward pyridoxal phosphate and the conservation of the lysine-HhH motif give seemingly contradictory information regarding Schiff's base formation. Selective Schiff's base formation for a abasic site-containing DNA would dictate that this enzyme functions through a specific interaction leading to catalysis unlike polyamines or lysine containing peptides. It will be of interest to determine how accurate such predictions concerning catalysis will be.

Acknowledgments. This work was supported by Grants GM52738 (G.P.M.) and ES063839 (S.H.W.) from the National Institutes of Health.

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(*Received* 14 *April* 1997; *revised* 1 *July* 1997; *accepted* 2 *July* 1997)