The Reaction Mechanism of DNA Glycosylase/AP Lyases at Abasic Sites†

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ABSTRACT: DNA glycosylase and glycosylase/abasic (AP) lyases are the enzymes responsible for initiating the base excision repair pathway by recognizing the damaged target base and catalyzing the breakage of the base−sugar glycosyl bond. The subset of glycosylases that have an associated AP lyase activity also catalyze DNA strand breakage at the resulting or preexisting AP site via a β-elimination reaction, proceeding from an enzyme−DNA imino intermediate. Two distinct mechanisms have been proposed for the formation of this intermediate. These mechanisms essentially differ in the nature of the first bond broken and the timing of the opening of the deoxyribose ring. The data presented here demonstrate that the combined rate of sugar ring opening and reduction of the sugar is significantly slower than the rate of formation of a T4-pyrimidine dimer glycosylase (T4-pdg)−DNA intermediate. Using a methyl-deoxyribofuranose AP-site analogue that is incapable of undergoing sugar ring opening, it was demonstrated that the T4-pdg reaction can initiate at the ring-closed form, albeit at a drastically reduced rate. T4-pdg preferentially cleaved the β-anomer of the methyl-deoxyribofuranose AP site analogue. This is consistent with a mechanism in which the methoxy group is backside-displaced by the amino group from the α-face of the deoxyribofuranose ring. In addition, studies examining rates of sugar−aldehyde reduction and the sodium borohydride concentration dependence of the rate of formation of the covalent imine intermediate suggest that the reduction of the intermediate is rate-limiting in the reaction.

DNA base excision repair is comprised of a series of discrete steps, including recognition of a damaged or inappropriate base, excision of this base, breakage of the phosphodiester backbone, resynthesis, and ligation. The glycosylase class of enzymes is responsible for initiating this repair pathway by recognizing the target base and catalyzing its removal via breakage of the glycosyl bond between the base and the sugar phosphate backbone (*1*, *2*). Excision of different bases thus leads to the production of a common intermediate in the pathway, the abasic (AP)[1] site or its imine equivalent. A subset of these glycosylases has an associated AP lyase activity which catalyzes strand breakage at an AP site via a β-elimination reaction, leaving a 3′-α, β-unsaturated aldehyde and a 5′-phosphate (*3*). A generalized mechanism has been proposed for the chemistry of the combined glycosylase/AP lyase activities in which the enzyme employs an amine as an active site nucleophile to attack the C1′ of the sugar associated with the damaged or inappropriate base, thereby forming an imino (Schiff base) intermediate (*4*). This reaction is characterized by several experimental hallmarks such as the ability to covalently trap the Schiff base intermediate by reduction with $NaBH_4$. This covalent enzyme−DNA complex can be experimentally isolated as a stable species (*5*−*7*).

The chemistry of the reactions leading to the formation of the Schiff base has been classified into two distinct mechanisms (Figure 1). These mechanisms differ in whether sugar ring opening is an enzyme-catalyzed step, closely coupled to formation of the Schiff base. In Figure 1, pathways 1.1, 1.2, or 1.3, ring opening is closely coupled to formation of the Schiff base. Alternatively, in Figure 1, pathway 2, the enzyme amine and an aldehyde, or its chemical equivalent, react by ordinary Schiff base formation. In the case of an AP site, the reactive species would be the C1′ aldehyde or its imine equivalent (*3*, *8*). In pathway 2, the aldehyde would be formed by non-enzyme-mediated sugar ring opening or by an earlier, distinctly separate, enzymatic step, not closely coupled to formation of the Schiff base. Pathway 1 can be further broken down into subschemes involving (pathway 1.1) protonation of the hydroxyl oxygen [modified base in the general case (*4*−*7*, *9*)], (pathway 1.2) electrostatic modulation of the electrophilicity of C1′, but no frank proton transfer to initiate the reaction (*10*), or

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[1] Abbreviations: AP, abasic; bp, base pair(s); BSA, bovine serum albumin; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DMAP, (dimethylamino)pyridine; FPG, formamidopyrimidine DNA glycosylase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PDG, pyrimidine dimer glycosylase; TBE, Tris borate−EDTA; UDG, uracil DNA glycosylase.

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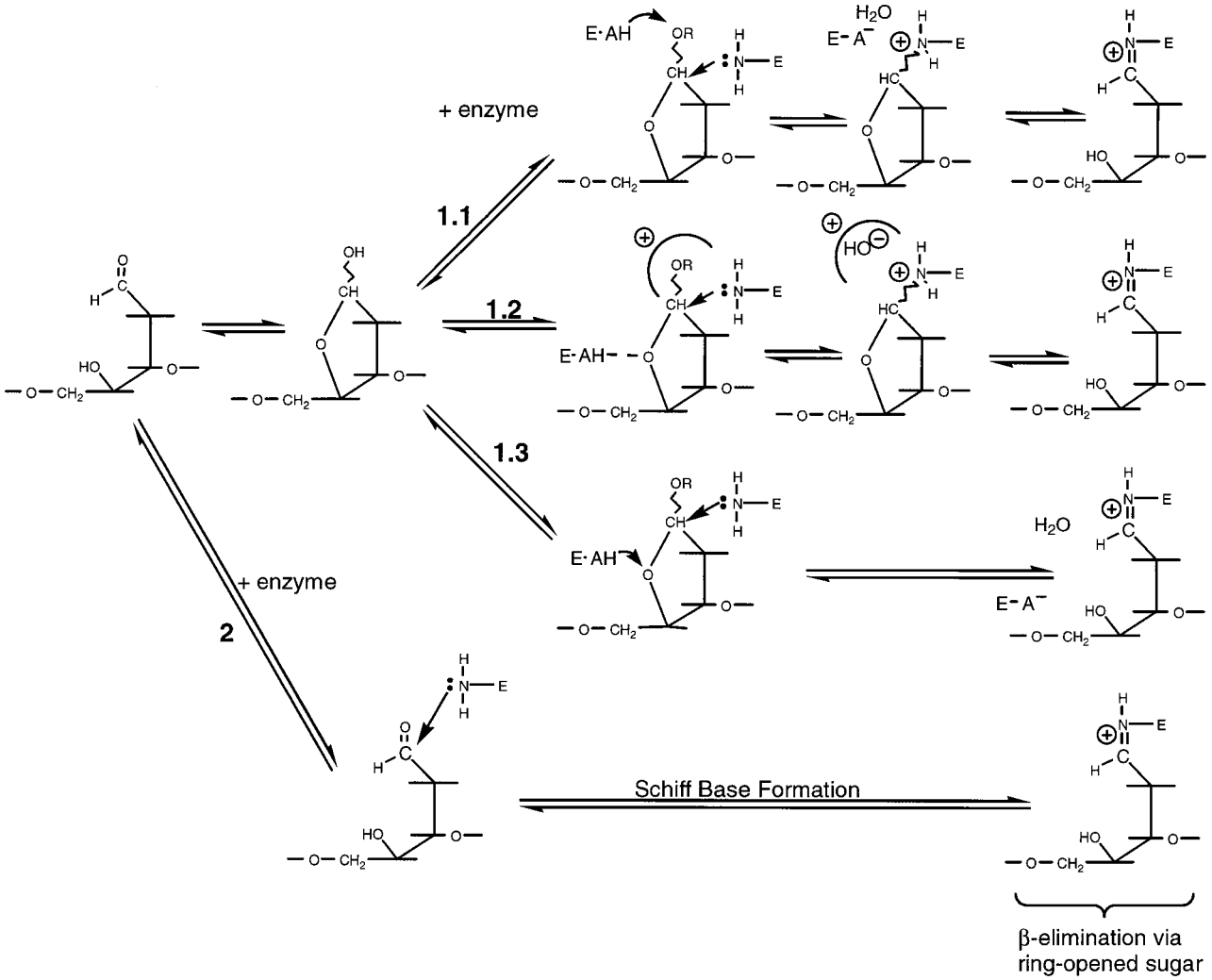

FIGURE 1: Initiation of glycosylase/AP lyase imine intermediate formation at hemiacetal (1) or aldehydic (2) AP sites or methyl-substituted AP sites. Schematic representation of possible pathways for the formation of the covalent protonated Schiff base intermediate formed between a DNA AP site (R═H) or the *O*-methyl-substituted AP site (R═CH_3) and a base excision repair glycosylase.

(pathway 1.3) protonation of the endocyclic sugar oxygen to initiate the coupled sugar ring opening and imino intermediate formation. The subsequent lyase step would proceed from the protonated Schiff base in any of these cases. Solution chemistry precedents have been investigated for pathways 1.1, 1.3, and 2 (*11*−*15*).

The AP sites resulting from glycosylase action exist in solution as a mixture of open chain aldehyde, hydrate, and cyclic hemiacetals. The ring-opened aldehydic form is present at less than 1% in solutions at equilibrium as determined by NMR; however, it is thought to be the most reactive of the AP site species (*16*). As discussed above, the formation of the Schiff base intermediate at an AP site may proceed through either the reactive aldehydic form or the more predominant hemiacetal forms (Figure 1). In these experiments, we utilize the ability to trap a covalent enzyme−DNA complex by reduction of the intermediate to investigate its formation by several well-characterized glycosylase/AP lyases and to distinguish among the proposed alternative mechanisms in Figure 1.

EXPERIMENTAL PROCEDURES

Materials. T4-pdg, cv-pdg, and *Escherichia coli* MutY were overexpressed and purified as described previously (*6*, *17*, *18*). *E. coli* endonuclease III, FPG, and AP endonuclease were generous gifts from T. O'Connor (City of Hope, Duarte, CA), B. Van Houten (National Institute of Environmental Health Sciences, Research Triangle Park, NC), and S. Mitra (University of Texas Medical Branch, Galveston, TX), respectively. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont-NEN and $NaBH_4$ from Fisher Scientific Co.

Uracil-Containing 49-mer Oligonucleotide. An oligonucleotide with the sequence 5′-AGCTACCATGCCTGCACGAA**U**TAAGCAATTCGTAATCATGGTCATAGCT-3′ was synthesized by Midland Research, and its complement was prepared by the NIEHS Center Molecular Biology Core (University of Texas Medical Branch, Galveston, TX). The U-containing oligonucleotide was γ-^{32}P-labeled on its 5′ end with T4 polynucleotide kinase and annealed to its complement following standard procedures. Labeled 49 bp U-containing duplex DNA was incubated for 15 min at 37 °C with uracil DNA glycosylase (0.25 unit/pmol of UDG, Epicenter Technologies) to prepare AP DNA substrates. Due to the inherent instability of AP DNA, all activity assays and trapping experiments were carried out immediately following preparation of the appropriate substrate(s).

The 12-mer sequence containing a centrally located TT pyrimidine dimer was GCACGAATTAAG where the un-

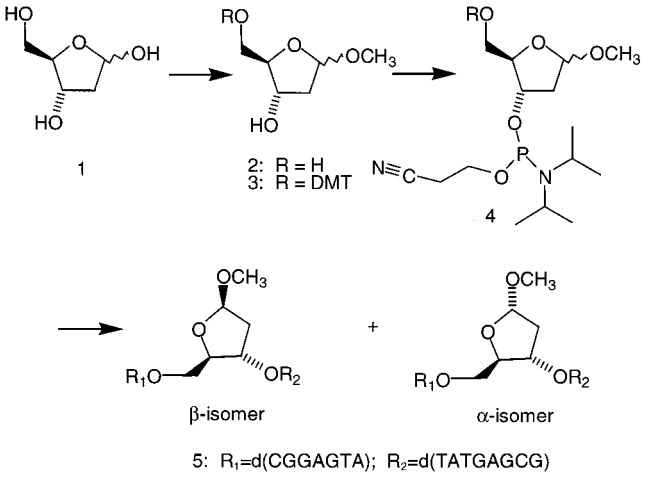

FIGURE 2: Synthetic scheme for the synthesis of the methyl deoxy-α- and -*β*-D-ribofuranose phosphoramidite building blocks.

derline represents the photodimer. The dimer-containing 12-mer was 5′-end-labeled and annealed to the complement strand as described above. Due to the relatively low melting temperature of this duplex, annealing and reactions were carried out at 4 °C.

Synthesis of Methyl 2-Deoxy-α- and -β-D-ribofuranose. (A) General Procedures. All reactions were carried out under a nitrogen atmosphere unless otherwise noted. All commercial materials were used without further purification unless otherwise stated. Anhydrous solvents were distilled from appropriate drying reagents prior to use. Analytical thin-layer chromatography was performed on Aldrich silica gel 60 F_{254} plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate−ammonium molybdate solution, followed by heating. Flash column chromatography was performed using the indicated solvent on E. Merck silica gel 60 (40−63 μm). NMR (^{1}H, ^{13}C, ^{31}P) spectra were recorded on a Varian XL-500 or 300 spectrometer and peaks referenced to acetone (^{1}H NMR, δ 2.10; ^{13}C NMR, δ 29.8), or 85% phosphoric acid (^{31}P NMR, δ 0.00) unless otherwise stated. IR spectra were recorded as a film on potassium bromide plates on a Mattson Instruments Polaris FT-IR spectrometer.

*(B) Methyl 5-O-(4,4′-Dimethoxytrityl)-2-deoxy-α- and -β-D-ribofuranose, **3**α and **3**β.* To 2-deoxy-D-ribofuranose **1** (5.0 g, Aldrich) (Figure 2) was added 0.05% methanolic hydrogen chloride solution (175 mL), and the mixture was stirred for 20 min at room temperature. Magnesium carbonate (2 g) was then added, and the suspension was vigorously stirred for 20 min. The reaction mixture was filtered and the solvent evaporated under reduced pressure to yield methyl 2-deoxy-α- and -*β*-D-ribofuranose **2**α and **2**β as colorless, mobile syrup in 90% yield (4.96 g). A portion of this product (1 g, 6.7 mmol) was dissolved in dry pyridine (20 mL), with dimethoxytrityl chloride (2.7 g, 8.1 mmol) and a catalytic amount of DMAP, and the mixture was stirred at room temperature for 3 h. The reaction was quenched with ice−water, and all volatile material was removed under vacuum. The residue was dissolved in dichloromethane and washed with saturated aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$, concentrated, and purified by flash chromatography on silica gel (eluent CH_2Cl_2/MeOH/Et_3N, 97:3:0.1; v/v/v), yielding the two diastereomeric products **3**α and **3**β in a 1:1.8 ratio. **3**α (minor): ^{1}H NMR (500 MHz, acetone-d_6): δ 7.53−7.48 (2H, m, ArH), 7.40−7.18 (7H, m, ArH), 6.91−6.85 (4H, m, ArH), 5.05 (1H, dd, J = 6, 2 Hz, H1), 4.12 (1H, m, H3), 4.07 (1H, m, H4), 3.75 (6H, s, $ArOCH_3$), 3.32 (3H, s, OCH_3), 3.19 (1H, m, H5), 3.13 (1H, m, H5) 2.32 (1H, ddd, J = 14, 8, 6 Hz, H2S), 1.83 (1H, ddd, J = 14, 4, 2 Hz, H2R); ^{13}C NMR (75 MHz, acetone-d_6): δ 159.4, 146.2, 136.9, 136.8, 130.8, 129.9, 128.9, 128.9, 128.6, 128.4, 128.2, 127.4, 127.4, 113.7, 113.5, 105.6, 85.3, 72.8, 64.9, 55.4, 54.7, 42.0; IR (Neat): 3444, 2923, 2830, 1616, 1504, 1240, 920 cm^{-1}; HRMS (FAB) calcd for $C_{27}H_{30}O_6$ (M^+) 450.2042, found 450.2021, 450.2046. **3**β (major): ^{1}H NMR (500 MHz, acetone-d_6): δ 7.56−7.50 (2H, m, ArH), 7.42−7.16 (7H, m, ArH), 6.91−6.85 (4H, m, ArH), 5.02 (1H, dd, J = 5, 2.5 Hz, H1), 4.31 (1H, m, H3), 4.17 (1H, d, OH), 3.98 (1H, m, H4), 3.77 (6H, s, $ArOCH_3$), 3.24 (3H, s, OCH_3), 3.16 (1H, m, H5), 3.13 (1H, m, H5), 2.04 (1H, ddd, J = 12.5, 7, 2.5 Hz, H2S), 1.97 (1H, ddd, J = 12.5, 7, 5 Hz, H2R); ^{13}C NMR (75 MHz, acetone-d_6): δ 159.5, 146.3, 137.2, 137.1, 130.9, 130.9, 129.0, 128.4, 127.3, 113.7, 105.8, 86.1, 72.4, 66.0, 55.4, 55.0, 42.2; IR (Neat): ν_{max} 3450, 2931, 2830, 1609, 1520, 1240, 910 cm^{-1}; HRMS (FAB) calcd for $C_{27}H_{30}O_6$ (M^+) 450.2042, found 450.2038, 450.2059.

*(C) Methyl 3-O-(Diisopropylamino-β-cyanoethylphosphoramidino)-5-O-(4,4′-dimethoxytrityl)-2-deoxy-α- and -β-D-ribofuranose, **4**α and **4**β.* The mixture of alcohols **3**α and **3**β (100 mg, 0.221 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phoshoramidite (100 mg, 0.33 mmol) were dissolved in dry acetonitrile (1.5 mL). 1*H*-Tetrazole (0.3 mmol, 0.67 mL; 0.45 mol dm^{-3} in acetonitrile) was added, and the mixture was stirred for 30 min at room temperature before being poured into 5% aqueous $NaHCO_3$ (15 mL) and extracted with dichloromethane (2 × 25 mL). The extracts were combined, dried ($MgSO_4$), and concentrated. Purification on a flash silica gel column (eluent hexane/ethyl acetate/triethylamine, 30:70:0.1, v/v/v) yielded the product mixture of **4**α and **4**β as a white foam (112 mg, 80%). ^{1}H NMR (300 MHz, acetone-d_6): δ 7.58−7.48 (2H, m, ArH), 7.44−7.18 (7H, m, ArH), 6.92−6.86 (4H, m, ArH), 5.13−5.05 (1H, m, H1′), 4.62−4.28 (1H, m), 4.20−4.00 (1H, m), 3.90−3.74 (1H, m), 3.79 (6H, s, $ArOCH_3$), 3.72−3.52 (3H, m), 3.36, 3.35 (1H, s, OCH_3), 3.29 (2H, s, OCH_3), 3.35−3.10 (2H, m), 2.84−2.68 (1H, m), 2.64−2.56 (1H, m), 2.54−2.41 (0.5H, m), 224−2.01 (1.5H, m), 1.34−1.02 (12H, m, *i*-PrH). ^{31}P NMR (121.5 MHz, acetone-d_6): δ major: 149.0, 148.9, minor: 148.8, 148.5.

*(D) Preparation of the Methyl 2-Deoxy-α- and -β-D-ribofuranose-Containing 16-mers **5**α and **5**β.* The 16-mer oligodeoxynucleotide was synthesized on an Applied Biosystems 380B DNA synthesizer on a 1 μmol scale and deprotected following standard procedures. Negative ion MALDI-TOF: calcd for M−H$^-$ 4863.842, found 4863.4. The sequence of this oligodeoxynucleotide was as follows:

5′-CGGAGTAXTATGAGCG-3′, where X is the methyldeoxyribofuranose. The complementary sequence was synthesized following standard procedures, and deprotected oligodeoxynucleotides were electrophoretically purified on a 15% denaturing polyacrylamide gel.

Formation of Covalent Enzyme−AP DNA Complexes Using $NaBH_4$. For general reactions, AP-containing DNA (1 nM) was incubated with 100 nM enzyme in 25 mM Na-

Hepes, pH 6.8, 100 mM NaCl, 100 μg/mL BSA, and 100 mM $NaBH_4$ for 5 min at 25 °C. Unless otherwise indicated, the enzyme and $NaBH_4$ were added simultaneously to the reaction by premixing the two. The activity of the enzyme was determined by incubating the DNA reaction mix with 100 nM enzyme for 5 min at 37 °C. Where indicated, pretreatment with $NaBH_4$ was for 5 min at 37 °C. For rate determinations, all reaction components were brought to 25 °C prior to mixing, and the reactions were carried out at 25 °C. $NaBH_4$ (30−100 mM) was added to the DNA in the reaction buffer described above, followed by the addition of 100 nM T4-pdg at the indicated times. The pH 9.0 reactions were carried out as above, except the reaction buffer consisted of 25 mM CHES, pH 9.0, 100 μg/mL BSA, 100 mM NaCl. An equal volume of loading buffer (95% v/v formamide, 90 mM Tris borate, 20 mM EDTA, 0.02% w/v bromophenol blue, 0.02% xylene cyanol) was added, and the samples were heated at 90 °C for 2−5 min prior to loading on a 15% polyacrylamide gel (8 M urea) in 1× TBE buffer (90 mM Tris borate, 2 mM EDTA, pH 8.0). The DNAs were separated by electrophoresis for 4 h at 20 W. Bands were visualized by autoradiography of wet gels using Hyperfilm-MP X-ray film (Amersham Corp.) and analyzed by a phosphorimager.

Prebound Enzyme Effect on $NaBH_4$ Reduction of AP Sites. AP-containing DNA (1.5 nM) was incubated as described above, with T4-pdg mutant E23Q for 15 min at 25 °C. Following binding of the enzyme, 100 mM $NaBH_4$ was added and subsequently loaded onto a G-25 Sephadex spin column to remove the $NaBH_4$. Three separate aliquots of the DNA were treated with either 70 nM T4-pdg, 70 nM AP endonuclease, or no enzyme for 30 min at 37 °C. Reactions were stopped and products analyzed as described above.

Enzyme Activity Assays. The methyl-deoxyribofuranose AP site-containing DNA was annealed at 4 °C. DNA (0.2 nM) was incubated with 100 nM enzyme in a standard reaction buffer (25 mM sodium phosphate, pH 6.8, 100 μg/mL BSA, 100 mM NaCl) in a total volume of 20 μL for 1−15 days at 4 °C. An equal volume of loading buffer was added, and the samples were analyzed as described above.

RESULTS

$NaBH_4$-Mediated Trapping of a T4-pdg−AP DNA Complex. Previously, it has been well documented that coincubation of DNA glycosylase/AP lyases with appropriate DNA substrates in the presence of a strong reducing agent leads to trapping of covalently bound complexes. In theory, it would be predicted that the reaction of DNA glycosylase/AP lyases with DNA containing an AP site would also lead to the formation of covalent DNA−enzyme intermediates, that can be reduced by $NaBH_4$. This hypothesis assumes that $NaBH_4$ reduction of the Schiff base enzyme−DNA intermediate is relatively fast, to yield enzyme bound to DNA, and that the reduction must take place after the enzyme has bound. Otherwise, the $NaBH_4$ would reduce those AP sites in the ring-open aldehydic form, and they would no longer be substrates for the enzyme reaction (*19*). As shown in Figure 3A, UDG treatment of the uracil-containing DNA (lane 2) resulted in complete conversion of the uracil to AP sites (lane 3) as evidenced by the conversion of 49-mer to 21-mer in the presence of T4-pdg (lane 4). Addition of $NaBH_4$ to the AP-containing DNA for 5 min prior to addition of the enzyme resulted in no detectable covalent complex formation (lane 6). This was due to reduction of the AP sites prior to enzyme addition or binding as evidenced by the lack of incision in these reactions. However, simultaneous addition of the enzyme with 100 mM $NaBH_4$ resulted in the formation of a covalent enzyme−DNA complex (>98%)-(lane 7). The small amount of reduced, uncleavable DNA that was remaining in these reactions (<2%) was most likely due to the equilibrium concentration of the ring-opened form which was susceptible to reduction by the 10^6-fold molar excess of $NaBH_4$. This interpretation is consistent with previous reports of the equilibrium fraction of AP sites existing as open chain aldehydes (*16*).

To demonstrate that the enzyme was effectively competing for the AP sites with $NaBH_4$, and not merely sterically blocking the aldehyde from reduction, the following analysis was carried out. The AP-containing DNA was incubated with a catalytically inactive mutant, E23Q, which retains full binding activity (*20*), prior to addition of excess $NaBH_4$. Prebinding of E23Q did not interfere with the reduction of the AP sites as evidenced by complete inhibition of cleavage by the subsequent addition of wild-type T4-pdg (Figure 3B, lane 5). AP-containing DNA was incubated with the $NaBH_4$ and T4-pdg simultaneously to form a covalent complex as shown in lane 8. E23Q bound at an AP site did not inhibit incision by wild-type T4-pdg in the absence of $NaBH_4$ (Figure 3B, lane 7), and neither $NaBH_4$ nor E23Q inhibited cleavage at the reduced AP site by AP endonuclease (lanes 3 and 6), an enzyme for which reduced AP sites are substrates. Thus, the presence of bound enzyme does not sterically interfere with the reduction of AP sites by $NaBH_4$.

Commonality in Schiff Base Intermediate Formation among Glycosylase/AP Lyases. To investigate if other DNA glycosylase/AP lyases initiate the formation of a Schiff base intermediate on an AP site, similar experiments were performed with several other well-characterized enzymes. As shown in Figure 4, simultaneous addition of enzyme with $NaBH_4$ resulted in covalent enzyme−DNA complex formation with no enzymatic cleavage for T4-pdg, cv-PDG, FPG, and endonuclease III (Figure 4, lanes 3, 5, 7, and 9). In the absence of $NaBH_4$, no covalent complexes were detected, and the substrate DNA was incised as shown by the disappearance of the substrate band (S) (lanes 2, 4, 6, 8). These results clearly demonstrated that the four glycosylase/AP lyases tested could all be covalently trapped at an AP site in the presence of $NaBH_4$ when added simultaneously with the enzyme. Interestingly, one of the enzymes tested, *E. coli* MutY, does not appear to form a covalent complex on AP site-containing DNA by these methods (Figure 4, lane 11) even though it exhibits AP lyase nicking activity (lane 10). These results can be rationalized by the structural and biochemical models of the MutY mechanism of action as suggested by the X-ray crystallographic structure (*21*). This analysis has implicated an opportunistically placed lysine residue (K142) near the active site as being responsible for Schiff base formation at AP sites generated by the glycosylase action, but the glycosylase reaction is chemically uncoupled from the lyase step. The identity of K142 as a residue capable of forming such an intermediate has been confirmed (*22*−*24*).

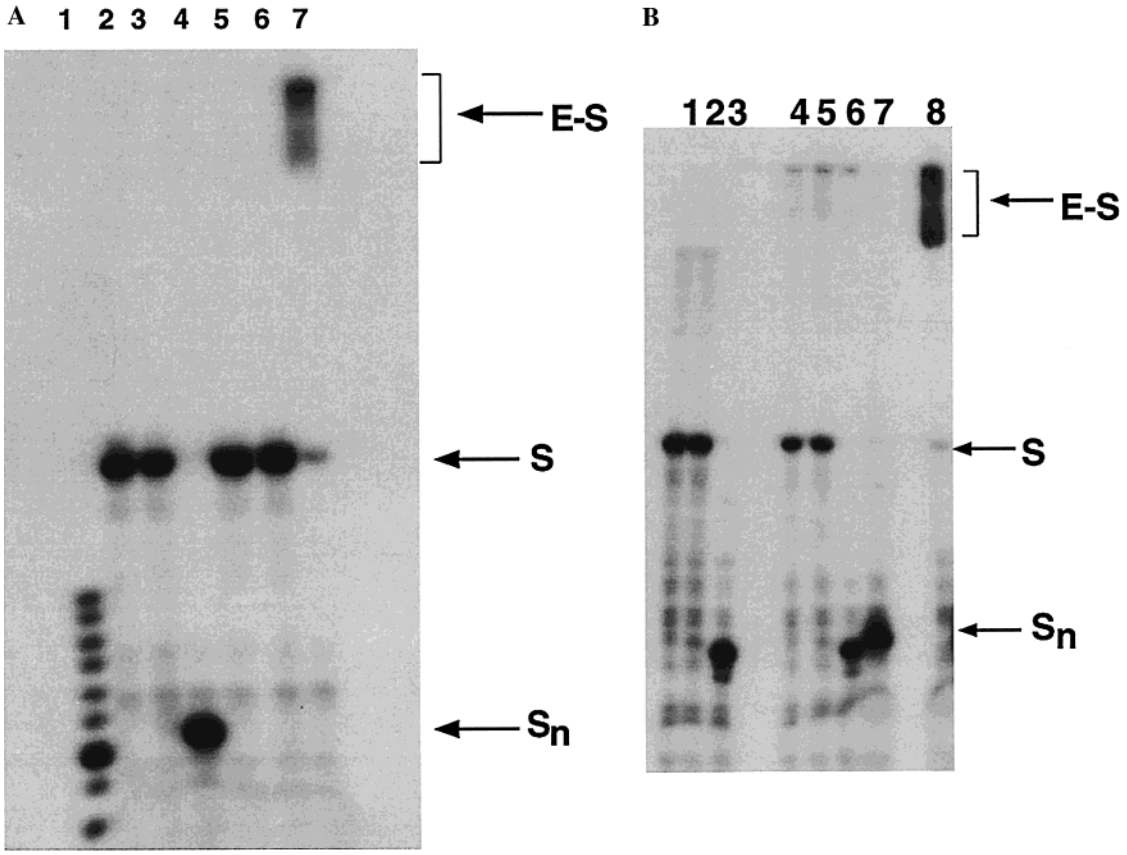

FIGURE 3: Formation of covalent enzyme−DNA complexes on DNA containing AP sites. Panel A: Formation of T4-pdg−AP DNA covalent complexes. 1 nM AP-containing DNA (lane 3) was incubated with 100 nM T4-pdg for 5 min at 25 °C (lane 4). 100 mM $NaBH_4$ was added either prior to (lane 6) or simultaneously (lane 7) with the enzyme. S = substrate; E−S = enzyme−substrate complex; Sn = nicked substrate. Lane 1, oligonucleotide markers 16−32 bases; lane 2, uracil-containing DNA; lane 5, AP DNA + $NaBH_4$. Panel B: Effect of prebound enzyme on $NaBH_4$ reduction of AP sites. 1.5 nM AP-containing DNA was incubated with T4-pdg mutant E23Q (lanes 4−7) or without enzyme (lanes 1−3) for 15 min at 25 °C. Then 100 mM $NaBH_4$ was added, and the samples were treated with either 70 nM T4-pdg (lanes 2 and 5), 70 nM AP endonuclease (lanes 3 and 6), or no enzyme (lanes 1 and 4) for 30 min at 37 °C. Lane 7 is AP-containing DNA incubated with 70 nM T4-pdg in the absence of $NaBH_4$ or E23Q. Lane 8 is AP DNA + T4-pdg and $NaBH_4$ added simultaneously.

Synthesis of Methyl Deoxyribofuranose-Containing DNA. The observation that covalent complex formation is possible upon simultaneous addition of enzyme and $NaBH_4$ to AP DNA suggested either (1) that the reaction is initiating at the hemiacetal form of the AP site, (2) that the conversion of the sugar from hemiacetal to an open aldehyde is slow, or (3) that the reduction of the aldehydic group by $NaBH_4$ is slow. To test the hypothesis that the glycosylase/AP lyase mechanism can initiate at the hemiacetal (closed sugar) predominant form of an AP site, we designed a 16 base pair oligodeoxynucleotide with an AP site analogue at position 8 from the 5′ end (Figure 2). In it, the hydroxyl at the C1′ position had been substituted by an *O*-methyl group. This glycoside cannot spontaneously open to the open-chain form and will not mutarotate. To incorporate the α- and *β*-anomers of methyl 2-deoxy-D-ribofuranose into an oligodeoxynucleotide by automated DNA synthesis, a phosphoramidite building block was synthesized from 2-deoxy-D-ribofuranose **1** in three steps as shown in Figure 2. In the first step, 2-deoxy-D-ribofuranose **1** was converted to a mixture of α- and *β*-anomers of methyl 2-deoxy-D-ribofuranose **2** by treatment with HCl in methanol. The 5′-hydroxyl of **2** was then protected with dimethoxytrityl chloride in methylene chloride according to standard procedures to give **3** as a 1:1.8 mixture of α- and *β*-anomers as determined by integration of the proton signals of the methoxy group at the anomeric carbon. The assignment was made by 2D NMR analysis of small samples of purified **3**α and **3***β*. In the COSY spectrum of the minor isomer, a cross-peak was observed between the anomeric methoxy group signal and the 3′-OH signal, which could only occur in the α-anomer via an intramolecularly H-bonded structure. In the NOESY spectrum of the minor isomer, cross-peaks were observed between one of the H2 proton signals and both the H1 and H3 signals, which would only be expected to occur for the α-anomer. Only a cross-peak between H1 and one H2 signal was observed in the NOESY spectrum of the major isomer. The coupling constants that were assigned for the major and minor products corresponded to the previously described coupling constants for **2***β* and **2**α and their derivatives (*25*). The shifts of the H2 protons of the major and minor isomers are quite similar to those reported for the major and minor isomers of the 5-*tert*-butyldiphenylsilyl derivative of methyl 2-deoxy-D-ribofuranose (*26*).

Cleavage of a Stable Ring-Closed AP Site Analogue. The 16-mer oligodeoxynucleotides were annealed at 4 °C due to inherent thermal instability of the duplex. This resulted in 100% duplex formation (data not shown) as measured by native gel electrophoresis. The duplex DNA was incubated with T4-pdg at 4 °C for up to 2 weeks. After 21 h incubation, the amount of product obtained for the methyl-deoxyribofuranose AP site analogue was 7% compared to 100%

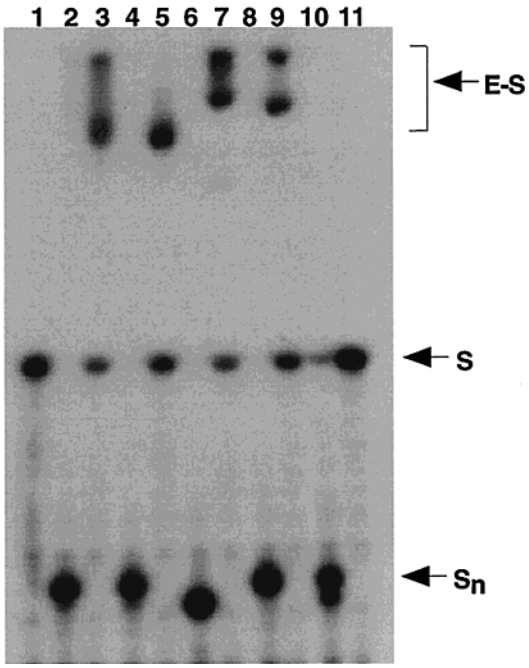

FIGURE 4: Covalent enzyme−DNA complex formation and enzyme activity on AP-containing DNA for several glycosylase/AP lyases. 1 nM AP-containing DNA was incubated with 100 nM enzyme in the presence (lanes 3, 5, 7, 9, 11) or absence (lanes 1, 2, 4, 6, 8, 10) of 100 mM $NaBH_4$ for 1 h at 37 °C. Lane 1, no enzyme; lanes 2, 3, T4-pdg; lanes 4, 5, cv-PDG; lanes 6, 7, FPG; lanes 8, 9, endonuclease III; lanes 10, 11, MutY.

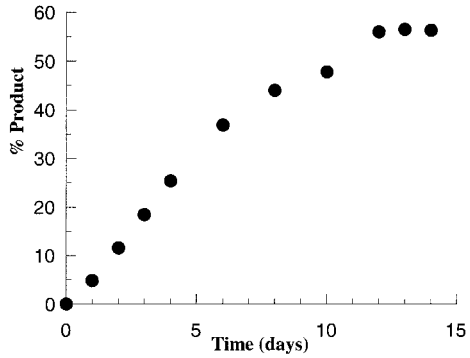

FIGURE 5: T4-pdg cleavage at a methyl deoxyribofuranose AP site analogue. 0.2 nM 5′-end-labeled duplex DNA was incubated with 100 nM T4-pdg in standard reaction buffer (25 mM sodium phosphate, pH 6.8, 100 mM NaCl, and 100 μg/mL BSA) for 15 days at 4 °C. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8 M urea) and analyzed as described in the text.

cleavage of a 12 bp DNA containing a TT-pyrimidine dimer. As seen in Figure 5, the amount of product obtained from cleavage of the methyl-deoxyribofuranose AP site analogue reached a maximum value at day 12 (56%). The absence of further cleavage is not due to instability of the enzyme over the 2 week period as T4-pdg is routinely stored at 4 °C for several years with no loss of activity. A maximum value of product at 56% indicates approximately 40% of the DNA is not cleavable. This may be explained by NMR data demonstrating that from the methyl-deoxyribofuranose AP site analogue synthesis a mixture of α- and β-anomers was obtained. It was determined that the ratio of these anomers in the mix is 1:2. These data are consistent with T4-pdg preferentially cleaving at the β-anomer.

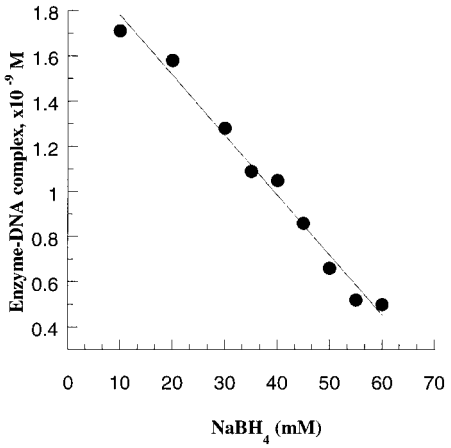

FIGURE 6: Effect of $NaBH_4$ concentration on formation of an AP DNA−enzyme complex. AP site-containing duplex DNA (1 nM) was preincubated with the indicated amounts of $NaBH_4$ (10−60 mM) for 20 s at 25 °C. 100 nM T4-pdg was added and the reaction stopped inmediately by addition of loading buffer and heating at 90 °C for 5 min. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8 M urea) and analyzed as described in the text.

$NaBH_4$ Concentration Dependence. The slow rate of cleavage of the methyl-deoxyribofuranose AP site analogue indicated that initiation of the AP lyase reaction at a hemiacetal is possible but probably represents a minor pathway with a high activation barrier. The other possible explanations for the initial observation of covalent trapping on AP sites, that the sugar ring anomerization to an open-aldehydic form is slow or that the reduction of the aldehyde is slow, were investigated. As we have no independent measure of sugar ring opening, the following assumptions were used. If the reduction step is fast, then the experimental rates would predominantly measure sugar ring opening. If the reduction is slow, then the reaction would be measuring the equilibrium concentration of the aldehyde form.

To determine the rate-limiting step in these reactions, we investigated the effect of $NaBH_4$ concentration on the extent and rate of reduction. These experiments were carried out by preincubating the AP DNA with various concentrations of $NaBH_4$ for a constant amount of time prior to the addition of enzyme. Thus, the addition of the enzyme and formation of the enzyme−DNA intermediate serve to effectively quench the sugar reduction reaction. Data shown in Figure 6 demonstrate that as the $NaBH_4$ concentration increases, the amount of covalent enzyme−DNA complexes formed decreases. This demonstrates a dependence of the rate on $NaBH_4$ concentration.

If the reduction is rate-limiting in the formation of the enzyme−DNA covalent complex, then there should be a correlative increase in the reaction rate as a function of increasing $NaBH_4$ concentration. As shown in Figure 7, upon simultaneous addition of the T4-pdg and the $NaBH_4$ to the DNA, 98% of all the AP-containing DNAs were covalently bound to the enzyme. Prior incubation of the DNA with the reducing agent for increasing times resulted in a decrease in the amount of covalent complexes observed. These data indicate that the average half-life of an AP site in the presence of $NaBH_4$ at pH 6.8 is approximately 12 s. At pH 9.0, the half-life of an AP site in the presence of $NaBH_4$ is 22 s (Figure 7).

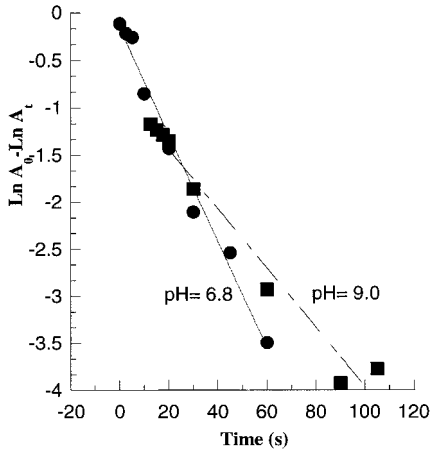

FIGURE 7: Rate of reduction of AP sites by $NaBH_4$. Plot shows experimental data obtained at two different pHs: (■) pH 9; (●) pH 6.8. 1 nM 5′-end-labeled 49 bp AP DNA was incubated with 100 nM T4-pdg in standard reaction buffer (25 mM HEPES, pH 6.8, 100 mM NaCl, and 100 μg/mL BSA or 25 mM CHES, pH 9.0, 100 mM NaCl, and 100 μg/mL BSA). Samples were incubated with 100 mM $NaBH_4$ at 25 °C for 20 s before adding the T4-pdg enzyme. Aliquots were taken at different times, and the reaction was stopped by addition of loading buffer and heating at 90 °C for 5 min. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8 M urea) and analyzed as described in the text.

Table 1: Sodium Borohydride Reduction: Pseudo-First-Order Rate Constants[a]

[$NaBH_4$], mM	k, s^{-1} (pH 6.8)	k, s^{-1} (pH 9.00)
30	Nd	0.021
50	Nd	0.024
100	0.06	0.031

[a] Rate constants were calculated by fitting the data to the first-order rate equation by a least-squares methods. Nd: No constants were determined at pH 6.8 for these $NaBH_4$ concentrations.

The pseudo-first-order rate constant increased with the concentration of $NaBH_4$ (Table 1). This indicates that these experiments measure the sum of two processes, ring opening plus reduction of the aldehyde form of the sugar ring with $NaBH_4$, with the reduction step being at least partially rate-limiting. Currently, there are no known independent rates of sugar ring opening within the context of DNA. In addition, the specific rate of reduction of an aldehydic moiety of an AP site by $NaBH_4$ is not known.

DISCUSSION

Our initial observation, that it is possible to isolate AP DNA covalent complexes of glycosylase/AP lyases in the presence of excess reducing agent, suggested to us that the formation of the Schiff base intermediate might be initiated through the enzyme reacting with the predominant cyclic form of the AP site (Figure 1, pathway 1). However, it was also possible that the interaction of the enzyme with the AP site was significantly faster than the reduction of the AP site by $NaBH_4$. Thus, the noncovalently enzyme-bound ring-opened sugar did not exist to an appreciable extent. Figure 1.1 illustrates sugar ring opening after initial protonation of the C1′ hydroxyl oxygen, while in contrast, Figure 1.3 depicts sugar ring opening initiated by protonation at O4′. The

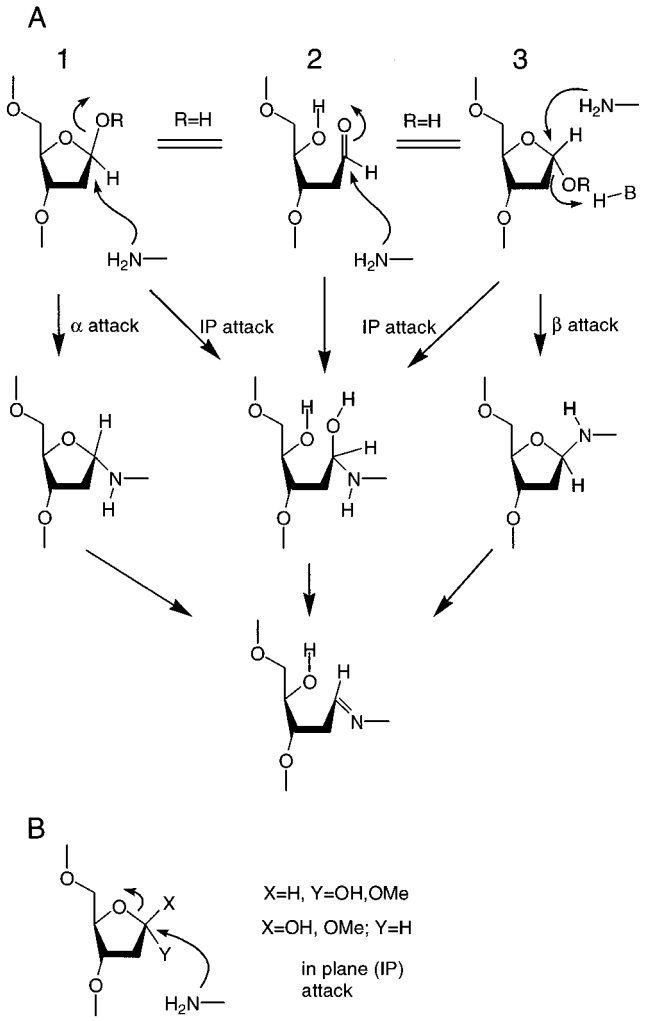

FIGURE 8: Nucleophilic amine attack geometric possibilities. This figure diagrams the geometric possibilities for the attacking amino group nucleophile of glycosylase/AP lyase enzymes. Pathway 1 shows that the amino group may attack from the α-face, leading to the ring-closed α-aminal, or in-plane (IP), leading, after dehydration, to the ring-opened Schiff base. Pathway 2 shows the formation of the Schiff base from the normal aldehyde and amino group reactants (corresponding to pathway 2 of Figure 1). Pathway 3 shows the amino group attacking from the β-face, leading to the ring-closed β-aminal, or an IP attack leading to the ring-opened Schiff base after dehydration.

hydroxyl or *O*-methyl (in the case of the analogue) at C1′ can be either α or β. The data shown in Figure 5 suggest that T4-pdg preferentially cleaves the β-anomer of the methyl-deoxyribofuranose AP site analogue. This is consistent with a mechanism in which the methoxy group is backside-displaced by the amino group from the α-face of the deoxyribofuranose ring (Figure 8A, pathway 1). This is the same direction of attack for direct displacement of the 5′-T of a thymine dimer by T4-pdg (*10*). The inability to cleave the α-anomer is also consistent with α-attack and not in-plane attack, which would have been expected not to discriminate between the α- and β-anomers. Thus, the experiments with the α- and β-anomers of methyl-deoxyribofuranose analogues indicate that, though very unfavorable compared to attack on the aldehyde (Figure 8A, pathway 2), the α-attack on C1′ is favored over the β-attack (Figure 8A, pathway 3) and in-plane attack, which is consistent with the mechanism of T4-pdg. These data also suggest that

protonation at O4′ (Figure 1, pathway 1.3) to initiate the reaction is unlikely as this would be independent of the α or β conformation.

It has been generally accepted in the prior literature that the ring-opened aldehydic sugar was the form involved in Schiff base formation leading to the lyase step (*27*, *28*). The results presented here suggest that the reaction at an AP site may also be initiated on the ring-closed (hemiacetal) sugar form, but that it is very slow. However, it is unclear whether this is an enzyme-catalyzed opening or if the enzyme "captures" the ring-opened form; these experiments cannot distinguish between the two. It is also unclear whether the reaction at an AP site proceeds along a reaction pathway closely analogous to that of the glycosylase reaction. One pathway to formation of a Schiff base intermediate at an AP site would be the reverse of the Schiff base hydrolysis (which would correspond to reaction 2 of Figure 1). Whether this pathway predominates over the reaction possibilities outlined in subreactions 1.1−1.3 of Figure 1 remains an open question. The fact that only the β-steric arrangement of the *O*-methyl at sugar C1′ was reactive with the enzyme strongly suggests that the in-plane attack possibilities diagrammed in Figure 8 do not occur at a substantial rate. Since the normal glycosylase step catalyzed by T4-pdg has the same geometric possibilities, either from the α-face or in-plane, an analogy with the reactivity of the methyl-deoxyribofuranose AP site analogue with the enzyme would strongly argue that the glycosylase step also consists of an attack from the α-face and not in-plane.

The E23Q preincubation data suggest that enzyme binding does not prevent reduction of the aldehydic form of the sugar and, thus, an enzyme-catalyzed ring opening tightly coupled to the formation of the Schiff base is likely. Under the reaction conditions used, it appears that the combined rates of deoxyribose ring opening and reduction of the sugar by $NaBH_4$ are slower than the rate of the enzyme binding to the substrate DNA by several orders of magnitude. A calculated rate of ring opening of a furanose ring as 58.8 s^{-1} (pH 6.2, at 25 °C) has been reported (*29*). This is faster than the process we are measuring, 0.06 s^{-1} at pH 6.8 at 25 °C (Table 1). Thus, ring opening may be 1000-fold faster than $NaBH_4$ reduction. It should be noted that this rate reflects the rate of furanose in solution and does not represent a sugar within the context of a DNA molecule. Thus, these rates may not be directly comparable. However, this difference suggests that the rate of reduction with $NaBH_4$ is the rate-limiting step.

In addition to providing some insight into the reaction mechanism for this class of enzymes, these experiments are important with regard to the technical implications of using the $NaBH_4$ trap as a quencher of reactions. If the formation of the imino intermediate is fast, such as T4-pdg, then the experiment may not be measuring a "snapshot" of the reaction but rather the rate of reduction.

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