The mechanism of the 5'-2-deoxyribose-5-phosphate lyase reaction catalyzed by mammalian DNA β-polymerase (β-pol) was investigated using a cross-linking methodology in combination with mass spectrometric analyses. The approach included proteolysis of the covalently cross-linked protein-DNA complex with trypsin, followed by isolation, peptide mapping, and mass spectrometric analyses. The approach included proteolysis of the covalently cross-linked protein-DNA complex with trypsin, followed by isolation, peptide mapping, and mass spectrometric analyses. The 8-kDa domain of β-pol was covalently cross-linked to a 5'-2-deoxyribose-5-phosphate-containing DNA substrate by sodium borohydride reduction. Using tandem mass spectrometry, the location of the DNA adduct on the 8-kDa domain was unequivocally determined to be at the Lys72 residue. No additional amino acid residues were found as minor cross-linked species. These data allow assignment of Lys72 as the sole Schiff base nucleophile in the 8-kDa domain of β-pol. These results provide the first direct evidence in support of a catalytic mechanism involving nucleophilic attack by Lys72 at the abasic site.

Mammalian DNA polymerase β (β-pol),1 a constitutively expressed “housekeeping” enzyme, has been implicated in DNA base excision repair (BER) (1–4). Base excision repair is thought to be the major repair pathway protecting cells against single base DNA damage. Recent evidence has indicated that BER in mammalian cells is mediated through at least two subpathways that are differentiated by the patch sizes and by the enzymes involved (5–8). These subpathways are designated as “single nucleotide” BER and long patch BER (two to five nucleotides). These two domains appear to be packed together in β-pol in solution, as revealed by comparison of axial ratios of β-pol (5.0) and the isolated 8- and 31-kDa domains (2.3 and 5.5, respectively) (17). Circular dichroism analysis has revealed that the 8-kDa domain is essentially α-helical in nature (16), and NMR solution and crystal structures (18, 19) have since confirmed the predominance of this secondary structure.

The 8-kDa domain of β-pol (residues 1–87), originally characterized as a single-stranded DNA binding domain, is formed from four α-helices. These helices are packed as two antiparallel pairs with 60° crossing between the pairs. Connecting segments between helices 1 and 2 and between helices 3 and 4 each contribute to DNA binding. Helix 3-turn-helix 4, which forms a “helix-hairpin-helix” motif is similar to the helix-hairpin-helix motif that has been found in a number of DNA repair proteins (18, 20, 21), including several DNA glycosylases and AP lyases (20, 22). Residues of the helix-hairpin-helix motif have been proposed to contribute to recognition and excision of damaged nucleotides in DNA, as well as AP lyase chemistry (23, 24). Alignment of the helix-hairpin-helix motifs from β-pol and endonuclease III suggests that Lys68 in β-pol may be important in lyase chemistry, because mutation of the analogous lysine residue in endonuclease III, Lys120, resulted in a dramatic reduction in AP lyase activity, and Lys120 has been proposed to be the Schiff base nucleophile in this enzyme (25).

In addition, the crystal structure of β-pol bound to a gapped DNA molecule representing a product of the dRP lyase reaction suggested that lysine residues 35 and 68 coordinate the 5'-phosphate that exists in the gapped DNA/enzyme crystal structure (Ref. 26 and Fig. 1).

Matsumoto and Kim (9) suggested that β-pol catalyzes removal of dRP from the AP endonuclease-incised AP site via β-elimination, as opposed to hydrolysis, and that this dRP lyase activity resides in the N-terminal 8-kDa domain of β-pol. In the β-elimination, the dRP excision reaction would proceed via an imine or Schiff base intermediate. Fisher et al. (27) demonstrated in 1958 that an imine intermediate formed between a substrate and enzymatic amino group can be trapped by reduction with sodium borohydride (NaBH₄). Since then this chemical technique has been widely used to elucidate reaction mechanisms, e.g. acetal decarboxylation (28) and aldolase (29).
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both cases, the ε-NH₂ group of a lysine was found to be involved in the formation of the imine intermediate. More recently, NaBH₄ trapping was used for the identification of imine intermediates in several DNA enzymes: bacteriophage T4 endonuclease V-DNA (30), Escherichia coli endonuclease III-DNA (31), E. coli Fpg-DNA (31, 32), and Micrococcus luteus UV endonuclease-DNA covalent complexes (33). More direct evidence that the 8-kDa domain of β-pol catalyzes removal of the dRP group via β-elimination was also obtained by Piersen et al. (12). These investigators showed that a Schiff base intermediate is formed between the dRP-containing DNA substrate and the enzyme. The Schiff base nuclease in the 8-kDa domain has been suggested to be Lys⁷² by site-directed mutagenesis (34, 35). This residue is in close proximity to the 5'-phosphate product group in the gapped DNA/enzyme crystal structure (26) and is part of the putative dyad lyase active site identified in NMR structures (23) of the 8-kDa domain (Fig. 1). Thus, based upon structural and site-directed mutagenesis data, it has been suggested that Lys⁷² is the preferred but not necessarily the obligatory residue in the dRP lyase active center of the 8-kDa domain of β-pol (24, 35, 36). To understand the precise mechanism of the lyase reaction catalyzed by β-pol and the lysine residue(s) involved in Schiff base chemistry, we utilized the NaBH₄ trapping technique in combination with mass spectrometric (MS) analysis. The 8-kDa domain was first covalently cross-linked to a dRP-containing DNA substrate by NaBH₄ reduction. We next identified the covalently modified lysine residue by MS sequencing. The approach included proteolysis of the covalently cross-linked protein-DNA complex with trypsin, followed by isolation, peptide mapping, and finally MS and MS/MS analyses of the adducted peptides. Our results, which unambiguously show that Lys⁷² in the 8-kDa domain of β-pol is the sole Schiff base nuclease, are discussed in the context of the structure of the dRP lyase active site.

EXPERIMENTAL PROCEDURES

Materials—The actinonitri le and ammonium bicarbonate (Fisher Scientific, Fair Lawn, NJ), trifluoroacetic acid (Ferred), formic acid (Al drich), and porcine trypsin (Promega Corporation, Madison, WI) were used as delivered. All buffers were prepared using water with a conductivity of 18-MΩ (Hydro Service and Supplies, Research Triangle Park, NC). Synthetic oligodeoxyribonucleotides purified by high pressure liquid chromatography were obtained from Oligos Etc, Inc. (Wilsonville, OR). [γ-³²P]ATP (3000 Ci/mmol), Mono S and Mono Q (HR 5/5) columns were purchased from Amersham Pharmacia Biotech. The recombinant 8-kDa domain of β-pol was overexpressed and purified as described previously (37). Human uracil-DNA glycosylase (UDG) with 84 amino acids deleted from the N terminus was purified as described (38).

5'-End Labeling—Deshphosphorylated 19-mer oligodeoxyribonucleotide (5'-UGTACCGATCCCCGGGTAC-3') containing a uracil residue at the 5'-end was phosphorylated with T4 polynucleotide kinase and [γ-³²P]ATP. The 54-mer (5'-GTCAGCTGCCGGGGATCCGTACCGCA-3') and 19-mer ³²P-labeled oligonucleotides by heating the solution at 90 °C for 3 min and allowing the solution to slowly cool to 25 °C. Unincorporated [γ-³²P]ATP was removed using a Nensorb-20 column according to the manufacturer’s suggested protocol. The ³²P-labeled duplex oligonucleotide, thus prepared, had a nick between positions 15 and 16. The radiolabeled oligonucleotide was lyophilized, resuspended in H₂O, and stored at −30 °C.

UDG Treatment of DNA Substrate—The ³²P-labeled duplex oligonucleotide was treated with human UDG that resulted in the ³²P-labeled deoxyxribose sugar phosphate at the nick. Typically, 50 nm DNA substrate was pretreated with 10 nm UDG in 50 mM Hepes, pH 7.4, 0.5 mM EDTA, and 0.2 mM dithiothreitol. The reaction mixture was incubated for 30 min at 37 °C. Due to the labile nature of the UDG-treated DNA, the DNA substrate was prepared just before performing the NaBH₄ trapping experiment.

Isolation and Purification of the 8-kDa Domain-DNA Complex—To prepare the covalently cross-linked 8-kDa DNA complex, the NaBH₄ trapping technique was used (12). Briefly, the reaction mixture (1 ml) containing 50 nm Hepes, pH 7.4, 0.5 mM EDTA, and 0.2 mM dithiothreitol was incubated at 37 °C for 30 min on ice. The reaction mixture was incubated for 30 min on ice and then 30 min at room temperature. After incubation, the reaction mixture was precipitated with ice-cold 10% (v/v) trichloroacetic acid. Under these conditions, the free protein and protein-DNA complex precipitate leaving the majority of the free DNA in the supernatant. The protein was then pelleted by centrifugation, washed twice with ice-cold 100% acetone, and air-dried. The pellet was solubilized in 8 M urea and diluted with 50 mM Tris-HCl, pH 8.8, to give a final urea concentration of 1 M. Subsequently, the sample was loaded onto an FPLC Mono S column (HR 5/5). The bound protein-DNA complex was eluted from the column using a NaCl gradient (0–1.0 M), and all fractions were collected and counted for radioactivity. Fractions with peak radioactivity were subjected to SDS-PAGE followed by autoradiography. At this stage, the fractions containing the protein-DNA complex were then pooled and digested with micrococcal nuclease (10 μg/ml). The covalently linked protein-DNA complex was precipitated with ice-cold trichloroacetic acid (10%), washed twice with ice-cold acetone (100%), and air-dried. The pellet was solubilized in 8 M urea and diluted with 50 mM (NH₄)₂CO₃ (pH 8.5) to a final urea concentration of 1 M. The protein-DNA complex was further purified using an FPLC Mono S column (HR 5/5). All fractions were counted for radioactivity and analyzed by SDS-PAGE and autoradiography. Fractions containing DNA-adducted protein were pooled and concentrated by trichloroacetic acid precipitation. Finally, the protein pellet was dissolved in 8 M urea and diluted with 50 mM (NH₄)₂CO₃ (pH 8.5) to a final urea concentration of 1 M.

The high performance liquid chromatography (HPLC) purification of the 8-kDa domain-DNA complex was performed by using a Hewlett-Packard Model 1100 HPLC system (Hewlett-Packard, Wilmington, DE) consisting of a Rheodyne 7125 sample injector (Rheodyne, Inc., Cotati, CA) with a 100-μl sample loop, a series 1100 binary pump, a series 1100
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Fig. 2. Optimization of covalent 8-kDa protein-DNA complex formation by NaBH₄ trapping. A 34-base pair oligonucleotide DNA containing [32P]dRP in the gap and the 8-kDa domain of β-pol were incubated with NaBH₄. Reaction conditions and product analysis are described under “Experimental Procedures.” A illustrates the duplex DNA (34 base pairs) that contained a solitary uracil at position 16, and a nick between positions 15 and 16. Uracil-containing oligonucleotide was 5'-end labeled, annealed, and treated with uracil-DNA glycosylase. DNA substrate, thus prepared, contained a 32P-labeled dRP in the gap and the 8-kDa domain of the protein-DNA complex. A dual-stage reflector mass spectrometer as has been previously described (41). A saturated MALDI matrix solution (0.5 μl) of α-cyano-4-hydroxycinnamic acid in 45:45:10 ethanol:water:formic acid and 0.5 μl of the sample solution were spotted onto the MALDI target. Co-crystallization of the sample and matrix was allowed to proceed at room temperature.

RESULTS

Optimization of Covalent 8-kDa DNA Complex Formation by NaBH₄ Trapping—To understand the mechanism of dRP lyase catalysis by the 8-kDa domain of β-pol and to identify the lysine residue(s) directly involved in Schiff base chemistry, NaBH₄ trapping (12) was used to isolate the imino complex formed between a DNA substrate and the 8-kDa domain. A duplex DNA (34 base pairs) that contained a solitary uracil at position

A 34-base pair oligonucleotide DNA containing [32P]dRP in the gap and the 8-kDa domain of β-pol were incubated with NaBH₄. Reaction conditions and product analysis are described under “Experimental Procedures.” A illustrates the duplex DNA (34 base pairs) that contained a solitary uracil at position 16, and a nick between positions 15 and 16. Uracil-containing oligonucleotide was 5'-end labeled, annealed, and treated with uracil-DNA glycosylase. DNA substrate, thus prepared, contained a 32P-labeled dRP in the gap and the 8-kDa domain of the protein-DNA complex. A dual-stage reflector mass spectrometer as has been previously described (41). A saturated MALDI matrix solution (0.5 μl) of α-cyano-4-hydroxycinnamic acid in 45:45:10 ethanol:water:formic acid and 0.5 μl of the sample solution were spotted onto the MALDI target. Co-crystallization of the sample and matrix was allowed to proceed at room temperature.

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16 and a nick between positions 15 and 16 was chosen as a matter of convenience; the uracil-containing oligonucleotide was 5'-end-labeled prior to annealing. Next, the duplex DNA was treated with uracil-DNA glycosylase to create a dRP-containing single nucleotide-gapped DNA substrate. The DNA substrate, thus prepared (12), contained a 32P-labeled dRP flap at the nick (Fig. 2A).

For preparation of large amounts of the covalent 8-kDa DNA complex, conditions for trapping the imino complex by NaBH₄ reduction were evaluated. Results depicted in Fig. 2B (lanes 1–3) show that when the 8-kDa and 32P-labeled DNA were used in varying ratios and incubated with NaBH₄ for 30 min at room temperature, little difference was detected in the amount of protein-DNA complex formed. Time course analysis of complex formation showed that a 30-min incubation of the 8-kDa protein and DNA with NaBH₄ gave results similar to longer incubations (Fig. 2B, lanes 4–6). For subsequent large scale preparations, isolation, and purification of the complex, 8-kDa

**Fig. 4. MALDI mass spectrum of the micrococcal nuclease-digested 8-kDa protein-DNA complex following purification by FPLC using a Mono S column.** MALDI spectra were acquired prior to HPLC purification (A) and after HPLC purification (B). Ions corresponding to the singly and doubly charged protein-DNA complex were observed at approximately 10,000 and 5,000 Da, respectively. The mass spectrum was acquired as described under “Experimental Procedures.” Ions labeled with an asterisk (*) correspond to background and/or micrococcal nuclease ions. The additional ion of m/z 9783 in B is most likely a decomposition ion due to loss of guanine.
protein and DNA were used in a 5:1 ratio (protein:DNA) and incubated for 30 min at room temperature.

Purification of the 8-kDa DNA Complex—The 8-kDa domain of β-pol contains 15 lysine and 4 arginine residues and has an isoelectric point of 10.3. Hence at pH 8.8, below the isoelectric point, the protein should have a net positive charge, whereas, after cross-linking to the DNA substrate, it should have a net negative charge. This charge difference was used to separate the covalently linked protein from the free 8-kDa protein on an FPLC Mono Q column. Under the conditions used (pH 8.8), free protein does not bind to the Mono Q column and emerges in the flow-through, whereas the cross-linked protein-DNA complex remains bound to the column. The bound protein-DNA complex was eluted from the column using a NaCl gradient. A small portion of each fraction was analyzed by SDS-PAGE. Fractions containing radiolabeled protein-DNA complex were pooled (Fig. 3, lane 1) and subjected to micrococcal nuclease digestion. Micrococcal nuclease digested the entire length of the DNA except for the covalently cross-linked nucleotide. Because most of the DNA was digested, the remaining protein-DNA complex should have a net positive charge and, hence, should bind to the Mono S column. FPLC Mono S column chromatography, therefore, was used to purify the protein-DNA complex after micrococcal nuclease digestion. Based upon SDS-PAGE analysis, the fractions that contained the protein-DNA complex were pooled (Fig. 3, lane 2) and used for subsequent MS and peptide map analyses.

Mass Spectrometric Analyses of the 8-kDa DNA Complex—Following micrococcal nuclease digestion and purification of the 8-kDa protein-DNA complex by FPLC using the Mono S column, the positive ion MALDI mass spectrum of the complex was acquired and is shown in Fig. 4A. The mass spectrum reveals ions corresponding to the protonated molecule of the protein-DNA complex at approximately 10,000 Da as well as ions that correspond in mass to protonated molecules of the micrococcal nuclease (ions labeled with an asterisk). Because the protein-DNA complex and the nuclease coelute from the Mono S column, the protein-DNA complex was further purified by reverse-phase HPLC using UV detection (Fig. 5A). As the DNA contains a 32P label, the fractions containing the 8-kDa protein-DNA complex were easily identified (Fig. 5B). The native 8-kDa domain protein, as well as the HPLC-purified protein-DNA complex, were then analyzed by both flow injection ESI/MS (Fig. 6) and MALDI/MS (Fig. 4B). The molecular mass of the 8-kDa protein-DNA covalent complex as determined by ESI/MS was 10,010 Da (Fig. 6B) in comparison to 9467 Da for the native 8-kDa protein (Fig. 6A). The mass accuracy of this instrument with external calibration is 0.01%, therefore, for molecular masses at 10,000 Da, the accuracy is ± 1 Da. These data suggest a molecular mass for the protein-DNA complex of 543 Da (± 1 Da) greater than that of the molecular mass of the protein (or less if the protein had become oxidized). Similar results were obtained from the MALDI/MS analysis (Fig. 4B).

LC/MS Analyses of the Tryptic Digests—To determine which amino acid(s) in β-pol interacts with the DNA 5'-phosphate, the 8-kDa domain alone and the 8-kDa domain-DNA complex were subjected to tryptic digestion and analysis by LC/ESI/MS. The potential tryptic cleavage sites in the amino acid sequence of the 8-kDa domain of β-pol and the resulting tryptic fragment numbers are shown in Fig. 7A. The mass chromatograms of the major tryptic fragments of the native 8-kDa protein were generated and compared with the mass chromatograms of these same tryptic fragments from the digest mixture of the protein-DNA complex. A notable difference observed between the mass chromatograms was that the relative abundance of tryptic fragment T15 (amino acids 73–81) was greatly reduced in the complex mixture in comparison to the native 8-kDa digestion mixture, indicating that the cross-linked DNA may be contained within these amino acid residues (data not shown). Note that the sample used for the tryptic digest of the 8-kDa protein-DNA also contained some residual native 8-kDa domain (Fig. 6B). It is, therefore, expected that tryptic fragments corresponding in mass to the native 8-kDa protein would be observed in the analysis of the digestion mixture of the 8-kDa protein-DNA complex. The most abundant new ion observed upon comparison of the LC/MS analysis of the digestion mixture of the complex (Fig. 7C) with that of the 8-kDa protein alone (Fig. 7B) was an ion ([M + 2H]2+ = 981.5) that eluted at 77.8 min and corresponds in mass to amino acid residues 69–81 (T14–15) plus 527 Da, in the protein-DNA complex digestion mixture, which was absent in the control digest mixture. The mass of this ion may correspond to the formation of the Schiff base intermediate followed by reduction. These data also indicate that 16 Da of the mass difference between the 8-kDa protein and the 8-kDa protein-DNA adduct are probably because of oxidation of one of the amino acid residues (i.e. mass increase of protein-DNA complex, 543 Da, less mass increase of adducted peptide, 527 Da, = oxygen, 16 Da). No signal was observed for the addition of 527 Da for any other predicted tryptic fragments above the baseline noise level (Table I). These data suggest that the DNA is cross-linked to one of the lysine residues in T14–15 (residues 69–81).

Determination of the Amino Acid in β-Pol Cross-linked to DNA—To determine whether the (M + 2H)2+ ion at m/z 981.5 (T14–15 plus 527 Da) contains the cross-linked DNA, an LC/MS/MS spectrum was acquired. The resulting MS/MS spectrum after transformation of all ions to the single charge state is shown in Fig. 8 and Table II. The major fragment ions are observed at m/z 1810.85, 1712.86, 1632.76, and 1534.79 (labeled as - G, - dG, - pdG, and - pdG - p) and correspond to the loss of guanine, deoxyguanosine, deoxyguanosine plus a phos-
phosphate, and deoxyguanosine plus two phosphate groups, respectively, from the peptide. These observed masses are within 0.05 Da of the calculated masses. These data confirm that the cross-linked DNA was contained within the T14–15 peptide. In addition, a series of both y and b ions (42, 43) are observed, which correspond to cleavages along the peptide backbone. The y series ions result from C-terminal peptide backbone cleavages and the b series ions result from N-terminal backbone cleavages. The y1 through y9 series ions correspond in mass to sequential loss of amino acids beginning at the C-terminal Lys81 and ending at Ile73. The b series ions (b4 - dG through b11 - dG) correspond in mass to cleavages of amino acids from the N terminus of the peptide backbone minus deoxyguanosine. The observation of the y10 - dG ion in addition to the observation of the y9 ion provides the necessary data to definitively assign the location of the DNA adduct. The observed mass difference between these two ions plus the mass of deoxyguanosine equals the mass of a lysine residue plus the mass of the DNA adduct. These structurally informative fragment ions allow the unequivocal assignment of the cross-linked DNA to the Lys72 residue of the 8-kDa domain of β-pol. No additional amino acid residues were found as minor cross-linked species (Table I).

**DISCUSSION**

In the present study, the mechanism of the dRP lyase reaction of human DNA β-polymersase has been investigated. To study the mechanism, the 8-kDa domain was covalently cross-linked to dRP-containing DNA by NaBH4 reduction, and the trapped intermediate was purified and then subjected to peptide mapping and MS sequencing analyses. Identification of the precise location of the DNA adduct on the 8-kDa protein has provided the first direct evidence in support of a catalytic mechanism involving nucleophilic attack by Lys72 at the abasic site. Based upon earlier site-directed mutagenesis studies (24, 34–36), Lys72 was shown to be involved at the dRP lyase active center of the 8-kDa domain of β-pol, but the precise role of Lys72 could not be assigned. Other residues that potentially could be involved in Schiff base formation include Lys35, Lys60, Lys68, and Lys84 (Fig. 1). To determine precisely the amino acid residue(s) covalently cross-linked to the DNA, the 8-kDa protein-DNA complex was subjected to tryptic digestion followed by mass spectrometric sequencing. Because of the complexity of the digestion mixture, on-line LC was used in conjunction with the MS analyses. The results of the LC/MS analysis of the tryptic digest of the 8-kDa protein-DNA complex were com-
pared with the LC/MS analysis of the tryptic digest of the native 8-kDa protein. For the LC/MS of the digest of the native 8-kDa domain, 91% of the protein sequence was detected. The tryptic digest ions corresponding to the remaining 9% of the protein had molecular masses that were below the effective mass range scanned for this particular experiment because of ions from chemical background at low mass.

LC/MS analysis of the tryptic digest of the 8-kDa protein-DNA complex showed the same tryptic digest ions that were observed in the LC/MS analysis of the native 8-kDa protein (data not shown). This observation is not surprising given the fact that some residual native 8-kDa protein was present in purified 8-kDa protein-DNA complex (Fig. 6B). In addition to the observation of the methionine-containing tryptic fragment ions T3–4 and T4, the oxidized form of these ions were also observed (data not shown). Based on ion counts, over 80% of tryptic fragment T4 appears to be oxidized. This indicates that the protein-DNA complex had become oxidized during the isolation and purification procedures. This confirms that the mass difference attributable to the DNA adduct is most likely 527 not 543 Da.

Mass chromatograms of ions corresponding to the addition of 527 Da to the tryptic fragments containing the suspected amino acid residues involved in dRP lyase activity based on mutagenesis studies were generated from the LC/MS data of the digests of both the protein-DNA complex and the native 8-kDa protein (Table I). The mass chromatogram corresponding to the doubly charged ion of tryptic fragment T14–15 plus 527 Da (m/z 981.5) from the LC/MS analyses of the tryptic digest of the native 8-kDa protein as a control and the purified 8-kDa protein-DNA complex are shown in B and C, respectively. The LC/MS analyses are described under “Experimental Procedures.”

**TABLE 1**

| Potential residue | Tryptic peptide AA residues | Tryptic fragment plus DNA adduct mass | Native 8-kDa protein | 8-kDa protein-DNA complex |
|-------------------|-----------------------------|--------------------------------------|----------------------|--------------------------|
| Lys35             | 26–40                       | 2089.88                              | NF                   | NF                       |
| Lys60             | 55–61                       | 1216.45                              | NF                   | NF                       |
| Lys68             | 62–72                       | 1638.74                              | NF                   | NF                       |
| Lys72             | 69–81                       | 1960.86                              | NF                   | F                        |
| Lys84             | 84–87                       | 1029.36                              | NF                   | NF                       |

*Note that trypsin would not cleave at a modified Lys, therefore, these peptides represent missed cleavage sites. AA, amino acid.

*Mass of neutral molecule.*

*NF, not found.*

*F, found.*

pared with the LC/MS analysis of the tryptic digest of the native 8-kDa protein. For the LC/MS of the digest of the native 8-kDa domain, 91% of the protein sequence was detected. The tryptic digest ions corresponding to the remaining 9% of the protein had molecular masses that were below the effective mass range scanned for this particular experiment because of ions from chemical background at low mass.

LC/MS analysis of the tryptic digest of the 8-kDa protein-DNA complex showed the same tryptic digest ions that were observed in the LC/MS analysis of the native 8-kDa protein
deoxyguanylic acid. Because no signal was observed for the addition of 527 Da to any of the other potential amino acid residues, these data suggested that the DNA adduct is covalently cross-linked to one of the amino acid residues 69–81.

To verify these results, the LC/MS/MS spectrum of the doubly charged ion of the T14–15 peptide was acquired. The tandem mass spectrum of the (M+H)²⁺ ion of m/z 981.5 in the tryptic digest mixture of the 8-kDa protein-DNA complex showed structurally informative fragment ions indicating the location of the DNA adduct on the tryptic peptide. After transformation of all ions to the single charge state, the resulting MS/MS spectrum showed abundant fragment ions corresponding to cleavages of the DNA that was adducted to the tryptic peptide (Fig. 8 and Table II). These ions included the loss of guanine (-G), loss of deoxyguanosine (-dG), loss of deoxyguanosine plus a phosphate (-pdG), and loss of deoxyguanosine plus two phosphates (-pdG - p). These data are consistent with the formation of a DNA adduct, which would contain an abasic site. In addition, a nearly complete series of C-terminal ions (i.e. y₁ to y₁₀) were observed, which correspond to the amino acid sequence IDEFLATGK. N-terminal ions minus the deoxyguanosine were also observed (i.e. b₁ - dG to b₁₁ - dG). The observation of these structurally informative fragment ions, most importantly y₉ and y₁₀ - dG, allows for definitive identification of Lys⁷² as the amino acid in the 8-kDa domain, which has been modified by covalent cross-linking to the DNA. Based upon the LC/MS and LC/MS/MS results, a proposed structure for the intermediate involved in dRP lyase activity is shown in Table II. The sole Schiff base intermediate in this enzyme is formed between the abasic site and the Lys⁷² residue of the 8-kDa domain.

In summary, using various purification and MS sequencing methodologies, the Schiff base intermediate trapped by reduction was identified. Tandem mass spectrometry provided structural information as to the location of the DNA adducted to the 8-kDa domain of β-pol. The amino acid residue located at the center of the lyase activity was unequivocally determined to be Lys⁷².
Lys72 of the 8-kDa domain of β-pol. Thus, peptide mapping in combination with mass spectrometry is an extremely powerful technique for investigating the structure of covalent intermediates in protein-DNA interactions.

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