Two Glycosylase/Abasic Lyases from Neisseria mucosa That Initiate DNA Repair at Sites of UV-induced Photoproducts*

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Diverse organisms ranging from Escherichia coli to humans contain a variety of DNA repair proteins that function in the removal of damage caused by shortwave UV light. This study reports the identification, purification, and biochemical characterization of two DNA glycosylases with associated abasic lyase activity from Neisseria mucosa. These enzymes, pyrimidine dimer glycosylase I and II (Nmu-pdg I and Nmu-pdg II), were purified 30,000- and 10,000-fold, respectively. SDS-polyacrylamide gel electrophoresis analysis indicated that Nmu-pdg I is approximately 30 kDa, whereas Nmu-pdg II is approximately 19 kDa. The N-terminal amino acid sequence of Nmu-pdg II exhibits 64 and 66% identity with E. coli and Hemophilus parainfluenzae endonuclease III, respectively. Both Nmu-pdg I and Nmu-pdg II were found to have broad substrate specificities, as evidenced by their ability to incise DNA containing many types of UV and some types of oxidative damage. Consistent with other glycosylase/abasic lyases, the existence of a covalent enzyme-DNA complex could be demonstrated for both Nmu-pdg I and II when reactions were carried out in the presence of sodium borohydride. These data indicate the involvement of an amino group in the catalytic reaction mechanism of both enzymes.

The exposure of DNA to UV light results in the formation of several photoproducts including cyclobutane pyrimidine dimers, (6-4) photoproducts, and Dewar photoproducts (1, 2). Cyclobutane pyrimidine dimers are the most common lesions produced by exposure of DNA to short wavelengths of UV light (below 295 nm). This lesion can exist in two main forms as follows: the cis-syn isomer, which is the predominant form, and the trans-syn isomer, which is less common (3). The trans-syn dimer exists as two stereoisomers, trans-syn I and trans-syn II (4). A defect in the repair of pyrimidine dimers can be associated with several biological consequences, including cell death, mutagenesis, and potentially carcinogenesis. Prokaryotic and eukaryotic organisms possess elaborate mechanisms either for the removal of these lesions (nucleotide excision repair and enzyme-catalyzed photoreversal) or for damage avoidance (recombination), thus enhancing survival and minimizing mutagenesis (reviewed in Refs. 5 and 6).

In addition, various organisms and viruses possess a base excision repair (BER) mechanism for the removal of these lesions (Ref. 7 and reviewed in Refs. 8–10). The initial step in BER is carried out by enzymes known as DNA glycosylases, which recognize and remove the damaged base. Many glycosylases have concomitant abasic (AP) lyase activity and are known as glycosylase/AP lyase enzymes. The prototype of these is endonuclease V (since renamed T4-pdg), which was originally discovered in Escherichia coli that had been infected with the bacteriophage T4.

T4-pdg is a well characterized BER enzyme that is specific for cis-syn cyclobutane pyrimidine dimers. In addition, this enzyme has been shown to incise DNA at the sites of trans-syn II dimers (3, 7) and the hydroxyl radical-induced adduct, 4,6-diamino-5-formamidopyrimidine (11). The rate of incision at these lesions is approximately 1% that of the rate of incision at cis-syn dimers (3, 7, 11). As expected for a glycosylase/AP lyase enzyme, T4-pdg also recognizes and cleaves DNA containing abasic sites.

The active site nucleophile of T4-pdg has been identified as the α-amino group of N-terminal Thr-2 (12–14), with catalysis requiring the acidic residue Glu-23 (15, 16). Nucleophilic attack on the C1’ of the sugar linked to the 5’-pyrimidine of the dimer results in cleavage of the N-glycosyl bond. This process results in the formation of an amino enzyme-DNA intermediate, which can be trapped by treatment with a reducing agent such as sodium borohydride (14, 17). In the absence of strong reducing agents, the sugar-phosphate backbone is cleaved on the 3’ side of the abasic site sugar by a β-elimination mechanism (lyase reaction). These results in cleavage products with 5’-phosphate and 3’-α,β-unsaturated aldehyde termini, respectively (14, 17–19). Thus, T4-pdg catalyzes successive N-glycosylase and AP lyase reactions.

A few microorganisms, including Micrococcus luteus, have been shown to harbor cyclobutane pyrimidine dimer-specific glycosylase/AP lyase activities similar to those of T4-pdg. Two different genes encoding enzymes that are specific for the repair of cyclobutane pyrimidine dimers in M. luteus have been cloned. One of these genes encodes a 31-kDa protein (Mlu-pdg I) that bears sequence similarities to E. coli endonuclease III and MutY (20). The other gene encodes an 18-kDa protein (Mlu-pdg II) that exhibits 27% amino acid sequence identity with T4-pdg (8, 9, 21). Recently, the first eukaryotic homolog of T4-pdg was reported (7, 22). Cp-pdg is encoded by the Paramecium bursaria chlorella virus-1 (PBCV-1) that infects the green algae Chlorella, and exhibits a 41% amino acid sequence iden-

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1 The abbreviations used are: BER, base excision repair; AP, abasic; THF, tetrahydrofuran; DHU, 5,6-dihydouracil; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography.
tity with T4-pdg. Additionally, it shows conservation of the functionally important amino acid residues (7, 23, 24).

For the last few years, our laboratory has been systematically searching for enzymes that initiate BER at UV-induced lesions. This study describes the identification, purification, and initial characterization of two pyrimidine dimer-specific glycosylase/AP lyase enzymes from the bacterium, *Neisseria mucosa*.

**MATERIALS AND METHODS**

**Reagents**—Heparin-Sepharose and Mono S column matrices, as well as FPLC supplies, deoxyoligonucleotide sizing markers, and Kodak XAR and Hyperfilm (X-Omat) autoradiography films were obtained from Amersham Pharmacia Biotech. Prestained protein molecular weight markers and cellophane membranes were purchased from Bio-Rad. (γ-32P)ATP (6,000 Ci/mmol) was purchased from NEN Life Science Products. T4 polynucleotide kinase and DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA). Plasmid AL-1A was purified by the NIEHS Protein Chemistry Core Laboratory at University of Texas Medical Branch. The BCA Protein Assay Reagent Kit was purchased from Pierce. All other reagents were purchased from Sigma.

**Protein Purification**—All purification procedures were performed at 4 °C. Approximately 100 g of frozen *N. mucosa* cells were thawed and resuspended in 150 ml of buffer A (20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 25 mM NaCl, 10% (v/v) ethylene glycol). The cells were lysed using a French press cell (11000 pounds/square inch), and the cellular debris was removed by centrifugation at 8,000 rpm for 20 min in a Sorvall GSA rotor. The resulting supernatant was loaded onto a 500-ml heparin-Sepharose column that had been equilibrated with 0.05% trifluoroacetic acid. The bound proteins were eluted with a linear NaCl gradient ranging from 0.05 to 0.56% NaCl. As a control, duplex 49-mer DNA without a lesion was used to determine the average number of single-strand breaks introduced in the DNA population.

**Deoxyoligonucleotide Substrates**—The sequences of the deoxyoligonucleotide substrates contain a single site-specific UV lesion (cis-syn, trans-syn I, trans-syn II, (6-4) photoproduct, or Dewar photoproduct of thymididylyl(3′ → 5′)-thymidine, AP lyase, or reduced AP lyase) that had been purified to apparent homogeneity. The surviving mass fraction of form I DNA was used to determine the average number of single-strand breaks introduced in the DNA population.

**Preparation of DNAs Containing AP and Reduced AP Sites**—A single-stranded 49-mer containing a single uracil at position 21 (Midland Reagent Co.) with the following sequence, 5′-AGTCTACATCCTGTT-CAGAAGUTAAGCAATTCGTAATCATGGTCATAG-3′, was labeled on the 5′ end using [γ-32P]ATP and then annealed to its complementary oligonucleotide.

DNA glycosylase and 100 mM NaBH4 in reaction buffer. The resulting duplex was incubated with 1 unit of uracil DNA glycosylase from the bacterium, *Escherichia coli* at 37 °C for 30 min. The remaining sample (10 μl) was treated with 1.0 M piperidine, incubated at 90 °C for 30 min, and then evaporated to dryness in a vacuum microcentrifuge for 1 h. Following evaporation, the sample was resuspended in 40 μl of stop buffer just prior to electrophoresis. This treatment cleaves any residual AP sites left by the enzyme. All samples were separated by electrophoresis through a 15% polyacrylamide gel containing 8 M urea. Electrophoresis was performed at constant 20 watts for approximately 4 h. The wet gels were dried and then exposed to a Phosphorlmager (Molecular Dynamics). Edman degradation, which was carried out by the NIEHS Protein Chemistry Core Laboratory at University of Texas Medical Branch, was used to determine the amino acid sequence of each of the purified enzymes.

**Specific Activity Determination**—UV damage-specific nicking assays were performed as described previously (25). Briefly, purified covalently closed circular plasmid DNA was UV irradiated to introduce approximately 25 dimers per plasmid molecule. The irradiated DNA (0.5 μg) was incubated with appropriate dilutions of enzymes from the various chromatographic steps in a standard reaction buffer containing 25 mM NaH2PO4 (pH 6.8), 1 mM EDTA, 100 mM NaCl, 100 μg/ml bovine serum albumin in a total reaction volume of 20 μl. Samples were incubated at 37 °C for 45 min, and reactions were stopped by the addition of an equal volume of agarose gel loading buffer. Form I and II DNAs were separated by electrophoresis through 1% agarose gels. The gels were stained with ethidium bromide overnight, and their images were captured and analyzed using a VIBASEM system from Amersham.
incubated with the enzyme in the presence of NaBH₄. A second set of controls contained NaCl (100 mM) in place of NaBH₄. After 30 min, the reactions were terminated and the products separated by electrophoresis through a 15% polyacrylamide gel containing 8 M urea. The wet gels were analyzed by autoradiography.

RESULTS

Identification of Microorganisms Expressing Pyrimidine Dimer-specific Nicking Activities—One of the recent goals of our research has been to identify and characterize novel DNA glycosylases, glycosylase/AP lyases, and endonucleases that are specific for UV light-induced photoproducts. To this end, 108 bacterial cell-free extracts (courtesy of Dr. Richard Roberts at New England Biolabs, Inc.) were screened for their ability to incise a double-stranded deoxyoligonucleotide containing either a single cis-syn cyclobutane thymine dimer or a (6-4) photoproduct. Several extracts were found to possess pyrimidine dimer-specific nicking activity, including those derived from the following: N. mucosa, Neisseria sicca, Neisseria mucosa heidelbergensis, Bacillus sphaericus, Moraxella bovis, Hemophilus parainfluenzae, and Hemophilus gallinarum (data not shown). Interestingly, none of these cell-free extracts were found to harbor (6-4) photoproduct-specific glycosylase or glycosylase/AP lyase activity, even though this photoproduct is found to harbor (6-4) photoproduct-specific glycosylase or glycosylase/AP lyase activity, even though this photoproduct is

| DNA substrates | UV photoproduct-containing DNAs | AP site- and reduced AP site-containing DNAs | DHU-containing DNA |
|----------------|--------------------------------|--------------------------------------------|-------------------|
| 5’-AGCTACCCAGCTGCAGTAATTGCAAATTCGTAATCATGTGCTCATAGCT-3’<sup>a</sup> | 5’-AGCTACCCAGCTGCAGTAATTGCAAATTCGTAATCATGTGCTCATAGCT-3’<sup>b</sup> | 5’-ATTATGCCTGTAGTATCCCTCTGCGCCCTGGAACCDACCTCAACCTCTGGCCAC-3’<sup>d</sup> |

<sup>a</sup>The bold TT in this sequence indicates a cis-syn dimer.
<sup>b</sup>The bold TT is a trans-syn dimer.
<sup>c</sup>The bold U represents a uracil residue that was enzymatically removed by uracil DNA glycosylase in the presence or absence of NaBH₄ to generate a reduced AP site or AP site, respectively.
<sup>d</sup>The bold D represents the position of the DHU.

Purification of Pyrimidine Dimer-specific Nicking Activities from N. mucosa—In an attempt to isolate the UV lesion-specific nicking activity observed in N. mucosa, we obtained and lysed approximately 100 g of N. mucosa cells (gift of Dr. Peter Goldfarb, New England Biolabs, Inc.). Soluble proteins were subjected to the following four chromatographic steps: single-stranded DNA agarose and heparin-Sepharose column chromatography, followed by FPLC (Mono S) and reversed-phase HPLC. Throughout these procedures, fractions were assayed for pyrimidine dimer-specific nicking activity using either a duplex DNA 49-mer containing a single centrally located cis-syn thymine dimer (3) (Table I) or UV light-damaged plasmid DNA (25). The extent of purification after each chromatographic step was determined by silver staining of SDS-polyacrylamide gels. The protein(s) responsible for the observed pyrimidine dimer-specific nicking activity bound tightly to the single-stranded DNA-agarose column, eluting in a broad peak with approximately 600–800 mM NaCl. This affinity is very similar to what has been previously reported for T4-pdg (27) and Mu-pdg I (28). The fractions containing activity were subjected to heparin-Sepharose column chromatography. Analysis of the resulting fractions revealed that there was a broad elution of proteins with pyrimidine dimer-specific nicking activity (Fig. 1). No activity was measured on nondimer-containing DNA (data not shown). Preliminary analyses of the cis-syn dimer incision data revealed that enzymes containing β- and β,δ-elimination activities eluted between ∼300 and 450 mM NaCl and again between 600 and 750 mM NaCl, whereas an activity displaying the hallmarks of a hydrolytic endonuclease was observed to elute between 450 and 600 mM NaCl. These column fractions were reassayed at greater dilutions, and analyses of these data revealed three distinctive peaks of activity (data not shown); however, these data also reinforced the assertion that the low and high salt elution peaks generated a 3’ sugar ring opened, α,β-unsaturated aldehyde β elimination product, and a 3’-phosphate δ elimination product. The middle fractions were presumed to possess hydrolytic activity for two reasons. The DNA products co-migrated with an authentic 3’-OH standard, and the migration of the products did not change upon pipidine treatment.

Since there was suggestive evidence for multiple activities, the purification of these three proteins was continued separately. Heparin-Sepharose fractions corresponding to the first peak of activity (fractions 7–10) were pooled and applied to a Mono S FPLC column. The bulk of this pyrimidine dimer-specific activity was recovered in the flow-through. SDS-PAGE followed by silver staining indicated that a protein of approximately 30 kDa was responsible for the observed activity (Fig. 2A). After further characterization (see below), this protein was named Nmu-pdg I.

Heparin-Sepharose fractions corresponding to the third peak of activity (fractions 17–21, see Fig. 1) were pooled and subjected to Mono S FPLC. Following this step, several proteins remained, as evidenced by SDS-PAGE followed by silver staining. Specifically, a doublet at 19–20 kDa and a band at 6 kDa were present (data not shown). In order to purify the protein responsible for the pyrimidine dimer-specific nicking activity to homogeneity, the Mono S fraction with the highest activity was chromatographed by reversed-phase HPLC. Pyrimidine dimer-specific nicking activity was recovered, and SDS-PAGE analysis revealed that the enzyme had been purified to apparent homogeneity (Fig. 2B). This enzyme was found to have an apparent molecular mass of approximately 19 kDa and, after subsequent characterization, was named Nmu-pdg II.

The specific activity measurements of Nmu-pdg I and Nmu-pdg II at each step of the purification procedure are given in Tables II and III, respectively. Nmu-pdg I was purified approximately 30,000-fold, whereas Nmu-pdg II was purified approximately 10,000-fold.

Since the heparin-Sepharose fractions corresponding to the second peak of dimer-specific nicking activity (Fig. 1, fractions 11–16) contained a protein that was found to have a catalytic mechanism different than those of Nmu-pdg I and II, the purification and characterization of this protein, named Nmu-uve, will be described elsewhere.

N-terminal Amino Acid Sequence—Whereas attempts were
made to determine the N-terminal amino acid sequence of both Nmu-pdg I and II, data were only obtained for Nmu-pdg II. Sequential Edman degradation revealed the following sequence, ATPSDASLRLFEVQKMDALLQSFQSMQRIV. A Blast search of the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, revealed that the partial sequence of Nmu-pdg II had 64 and 66% amino acid identity with endonuclease III from E. coli and H. parainfluenza, respectively. These results are intriguing since endonuclease III is a well characterized glycosylase/AP lyase that has specificity for oxidized pyrimidine residues (19, 29). Endonuclease III has a high sequence homology with Mlu-pdg I, a pyrimidine dimer-specific glycosylase/AP lyase from M. luteus (20), and MutY, an adenine-specific mismatch glycosylase of E. coli (30).

Comparison of Nmu-pdg I, Nmu-pdg II, and T4-pdg Reaction Products—Reaction of a double-stranded deoxyoligonucleotide containing a single cis-syn thymine dimer with purified Nmu-pdg I, Nmu-pdg II, or T4-pdg yielded DNA cleavage products with similar electrophoretic mobilities (Fig. 3, lanes 5, 11 and 2, respectively). T4-pdg is known to incise the N-glycosyl bond of the 5’-pyrimidine of a dimer and then cleave the sugar-phosphate backbone on the 3’ side of the abasic site sugar by a β-elimination mechanism. Its cleavage product terminates in a 3’ α,β-unsaturated aldehyde and can be identified by gel electrophoresis (18, 19). Treatment of this product with piperidine results in a loss of the modified sugar, leaving a 3’-phosphate terminus. Thus piperidine treatment causes an increase in electrophoretic mobility of the reaction product. The mobilities of the cleavage products of Nmu-pdg I and II with (Fig. 3, lanes 6 and 12) and without (lanes 5 and 11) piperidine treatment appear identical to those of the cleavage products of T4-pdg with (lane 3) and without (lane 2) piperidine treatment. Thus, Nmu-pdg I and II appear to be pyrimidine dimer-specific glycosylase/AP lyases, yield similar cleavage products to that of T4-pdg, and have been named accordingly.

In contrast to Nmu-pdg I and II, the enzyme we have named Nmu-uve appears to yield cleavage products with mobilities distinct from those of the T4-pdg cleavage product (Fig. 3, lanes 8 and 9 and 2 and 3). Piperidine treatment of the Nmu-uve cleavage products caused no alteration in mobility (Fig. 3, lane 9), indicating that the 3’ terminus of the product prior to piperidine treatment is not an α,β-unsaturated aldehyde. Although the gel shown in Fig. 3 does not have the resolution to allow separation of products with 3’-OH and 3’-phosphate termini, additional studies (to be described elsewhere) indicate that the cleavage product of Nmu-uve terminates in a 3’-OH. This is consistent with a hydrolytic mechanism, hence the name Nmu-uve (for N. mucosa UV endonuclease).

Substrate Specificity—In order to determine the substrate specificities of Nmu-pdg I and II, assays were performed with DNAs containing various lesions. These lesions included cis-syn, trans-syn I, trans-syn II, (6-4) and Dewar photoproducts, an AP site, a reduced AP site, DHU, tetrahydrofuran (THF), and pyrrolidine. Nmu-pdg I exhibited a very broad substrate specificity (Fig. 4), as shown by its ability to nick oligonucleotides containing a cis-syn (lanes 16 and 17) or trans-syn I (lanes 2 and 3) thymine dimer, an AP site (lanes 10 and 11), or DHU (lanes 14 and 15). Very modest levels of incision were observed.

![Figure 1: Pyrimidine dimer-specific nicking activity of heparin-Sepharose fractions.](image1)

![Figure 2: Silver-stained gel of Nmu-pdg I and II.](image2)
the amount of form I DNA remaining was determined by BioImage analysis. The average number of single-strand breaks (SSB) per DNA molecule was calculated from the amount of form I DNA in 45 min at 37 °C.

(data not shown).

reactions lacking enzyme showed no cleavage at these sites. In contrast, when an oligonucleotide containing a reduced AP site was used as a substrate, no damage-specific incisions were observed (Fig. 4, lanes 11 and 12). No cleavage was observed using DNAs containing THF or pyrrolidine as substrates (data not shown).

Specific activity analyses for Nmu-pdg II

| Purification step | Total volume | SSB/DNA | Total units | Total protein concentration | Specific activity |
|-------------------|--------------|---------|-------------|-----------------------------|------------------|
| Lysate            | 275          | 0.9     | 2.5 × 10^5  | 4.7 × 10^4                  | 5                |
| Single-stranded DNA-agarose | 750 | 0.8    | 2.0 × 10^5  | 8.5 × 10^4                  | 2.4 × 10^7       |
| Heparin-Sepharose | 12           | 0.6     | 2.4 × 10^4  | 8 × 10^-1                   | 3.0 × 10^4       |
| Mono S            | 50           | 0.8     | 1.3 × 10^4  | 9 × 10^-2                   | 1.5 × 10^4       |

a UV-irradiated plasmid DNAs were incubated with an appropriate dilution of Nmu-pdg II at 37 °C for 45 min. After agarose gel electrophoresis, the amount of form I DNA remaining was determined by BioImage analysis. The average number of SSB per DNA molecule was calculated from the ln (mass fraction of form I DNA).

b Units are defined as the amount of enzyme required to introduce an average of one SSB/DNA molecule into 0.5 µg of UV-irradiated plasmid DNA in 45 min at 37 °C.

![Fig. 3. Nature of the 3' termini created by Nmu-pdg I, Nmu-pdg II, and Nmu-uv. A duplex 5'-end-labeled 49-mer oligonucleotide containing a single centrally located thymine dimer was incubated individually with Nmu-pdg I, Nmu-pdg II, and Nmu-uv at 35 °C. Samples were analyzed as described in Fig. 2. Lane 1, oligonucleotide sizing markers (8–32 nucleotides); lanes 2 and 3, T4-pdg without (lane 2) and with (lane 3) piperidine treatment; lanes 4, 7, 10, and 13, 5'-end-labeled 20-mer with a 3'-OH terminus; lanes 5 and 6, Nmu-pdg I without (lane 5) and with (lane 6) piperidine treatment; lanes 8 and 9, Nmu-uv without (lane 8) and with (lane 9) piperidine treatment; lanes 11 and 12, Nmu-pdg II without (lane 11) and with (lane 12) piperidine treatment; lanes 14–16, undamaged 49-mer DNA plus Nmu-pdg I (lane 14), Nmu-pdg II (lane 15), and Nmu-uv (lane 16).](image)

using DNA with (6-4) or Dewar photoproducts (lanes 8 and 9 and 6 and 7, respectively). On the substrates containing (6-4) or Dewar photoproducts, Nmu-pdg I appeared to function primarily as a DNA glycosylase, since cleavage was not evident until the reaction products were treated with piperidine. Control reactions lacking enzyme showed no cleavage at these sites (data not shown). Nmu-pdg I was unable to incise DNAs containing either a trans-syn II dimer (lanes 4 and 5) or a reduced AP site (lanes 12 and 13). Although the AP site-containing oligonucleotide exhibited some additional degradation products, accumulation of a labeled product of the expected size (20-mer) and the total disappearance of the substrate were indicative that Nmu-pdg I is highly reactive with AP site-containing DNA (Fig. 4, lanes 10 and 11). In contrast, when an oligonucleotide containing a reduced AP site was used as a substrate, no damage-specific incisions were observed (Fig. 4, lanes 12 and 13). No cleavage was observed using DNAs containing THF or pyrrolidine as substrates (data not shown).

Nmu-pdg II was also shown to have a broad substrate specificity (Fig. 5) and had the ability to recognize and nick DNA at
various lesions, including cis-syn and trans-syn I thymine dimers (lanes 2 and 3), an AP site (lanes 10 and 11), and DHU (lanes 14 and 15). Similar to Nmu-pdg I, this enzyme shows weak glycosylase activity on (6-4) (lanes 8 and 9) and Dewar photoproducts (lanes 6 and 7), and DNA cleavage was only observed after piperidine treatment. Although Nmu-pdg II appears to function primarily as a combined glycosylase/AP lyase on the DHU-containing substrate (Fig. 5, lanes 14 and 15), Nmu-pdg I appears to function mainly as a glycosylase on this substrate, since the cleavage product is much more prevalent after piperidine treatment (Fig. 4, lanes 14 and 15). Like Nmu-pdg I, AP site-containing DNA was a very good substrate for Nmu-pdg II, as evidenced by all the substrate being converted to the expected size product (lanes 10 and 11). DNAs containing a reduced AP site or trans-syn II thymine dimer were not substrates for Nmu-pdg II (lanes 12 and 13 and 4 and 5, respectively). Furthermore, no cleavage was observed using DNAs containing THF or pyrrolidine as substrates (data not shown).

Trapping the Covalent Enzyme-DNA Intermediates—Glycosyl bond scission at a DNA lesion such as a pyrimidine dimer can occur by an initial attack by a nucleophile on C1' of the 5' sugar linking the lesion to the phosphodiester backbone. This nucleophile can either be a primary amino group or an activated water molecule (17). If the attacking nucleophile is a primary amino group, a Schiff base intermediate results, which can be reduced by NaBH4. This chemical reduction, while the intermediate is still present, will result in a stable covalent linkage between the enzyme and the DNA that can be detected as a shifted band by gel electrophoresis (14, 17, 31). Conversely, if the attacking nucleophile is an activated water molecule, no enzyme-DNA covalent intermediate would be formed and thus no shifted band would be observed.

Reaction of Nmu-pdg I with cis-syn dimer-containing DNA in the presence of NaBH4 followed by gel electrophoresis revealed the presence of a specific enzyme-DNA complex (Fig. 6A). As the amount of enzyme was increased, the intensity of the shifted band increased (Fig. 6A, lanes 3–6), indicating dose dependence of this reaction. T4-pdg, which is known to form a stable enzyme-DNA complex with this substrate in the presence of NaBH4 (13), was used as a positive control (Fig. 6A, lane 2). No complexes were detected using dimer-containing DNA in the absence of enzyme or with undamaged DNA in the presence of enzyme (lanes 1 and 7, respectively).

As with Nmu-pdg I, Nmu-pdg II was found to form a stable enzyme-DNA intermediate in the presence of NaBH4 using
cis-syn dimer-containing DNA as the substrate (Fig. 6B). Again, T4-pdg was used as a positive control (lanes 1–3). The intensities of the shifted bands appeared to be directly proportional to the amount of Nmu-pdg II (Fig. 6B, lanes 4–6) present in each reaction. No covalent enzyme-DNA complex was detected using either undamaged DNA in the presence of sodium borohydride. This enzyme attacks C1' of the 5' sugar attached to the dimer.

In this work, Nmu-pdg II was purified to apparent homogeneity and was found to be a protein of approximately 19 kDa (Fig. 2B). Nmu-pdg II exhibited maximal activity over a pH range of 5.5–7.2, peaking at pH 6.0 (data not shown). The N-terminal amino acid sequence of Nmu-pdg II revealed a 64% amino acid identity with E. coli endonuclease III and a 66% identity with H. influenza endonuclease III. Nmu-pdg II represents the second member of the endonuclease III family that has UV lesion-specific nicking activity, the first being MutY (20). However, in contrast to MutY-pdg, Nmu-pdg II has been shown to nick DNA-containing DHU, a lesion formed by oxidative damage. Interestingly, this lesion is a substrate for endonuclease III.

Endonuclease III has been shown to flip its target nucleotide extrahelically and to utilize a primary amino group as the attacking nucleophile (32, 33). Since endonuclease III shares significant amino acid sequence homology with E. coli MutY and Mlu-pdg, which also have been shown to utilize a primary amine as the attacking nucleophile (20, 30), it may be plausible to propose that Nmu-pdg II uses a similar catalytic and nucleotide flipping mechanism. Proof of this proposal awaits cloning, expression, and structural determination of Nmu-pdg II.

Nmu-pdg I was purified approximately 30,000-fold to a very high specific activity (Table II) and is a protein of approximately 30 kDa (Fig. 2A). The pH activity profile for Nmu-pdg I ranges from pH 5.5 to 7.5, peaking between pH 6.0 and 7.2 (data not shown). Because we were not able to sequence this protein, it remains to be determined whether or not Nmu-pdg I has amino acid sequence homology with other known glycosylase/AP lyases. The possibility also exists that the 19-kDa Nmu-pdg II is the catalytically competent domain of the 30-kDa enzyme, Nmu-pdg I enzyme.
The ability of Nmu-pdg I and II to initiate the removal of trans-syn I and not trans-syn II dimers is interesting because the two photoisomers differ from one another and from the cis-syn dimer only in the configuration of the thymines. The trans-syn I dimer has the 5′-thymine of the dimer “flipped out” of the plane of the normal base stacking, whereas the trans-syn II dimer has the 3′-thymine of the dimer flipped out of the plane of the base stacking (7). The results of the substrate specificity for Nmu-pdg I and II suggest that the substrate recognition mechanisms employed by these enzymes can discriminate between the two configurations. In contrast to Nmu-pdg I and II, T4-pdg and cv-pdg were able to incise DNA containing the trans-syn II dimer but neither incises DNA containing a trans-syn I lesion (7). Biophysical studies will be essential in determining the structural basis of these substrate specificity differences. The solution of a crystal structure of each of these enzymes in a complex with an uncleavable DNA substrate may reveal the nature of their specific enzyme-substrate interactions. This could be useful in explaining how the trans-syn I dimer serves as a substrate for Nmu-pdg I and II, whereas the trans-syn II dimer does not.

At this time, it is unclear why N. mucosa has such redundancy in enzymes to initiate base excision repair at sites of UV damage. It may be reasonable to speculate that the broad substrate specificities of the three enzymes contribute to their conserved nature.

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