Purification and Cloning of Micrococcus luteus Ultraviolet Endonuclease, an N-Glycosylase/Abasic Lyase That Proceeds via an Imino Enzyme-DNA Intermediate*

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Although Micrococcus luteus UV endonuclease has been reported to be an 18-kDa enzyme with possible homology to the 16-kDa endonuclease V from bacteriophage T4 (Gordon, L. K., and Haseltine, W. A. (1980) J. Biol. Chem. 255, 12047-12050; Grafstrom, R. H., Park, L., and Grossman, L. (1982) J. Biol. Chem. 257, 13465-13474), this study describes three independent purification schemes in which M. luteus UV damage-specific or pyrimidine dimer-specific nicking activity was associated with two proteins of apparent molecular masses of 31 and 32 kDa. An 18-kDa contaminant copurified with the doublet through many of the chromatographic steps, but it was determined to be a homolog of M. luteus protein inactivated on cis-syn thymine dimers; it was unable to cleave site-specific oligonucleotide substrates containing a trans-syn-dimer, (6-4), or Dewar thymine dimer, a 5,6-dihydropurine-cytosine lesion, or an A:C or A:T mismatch. The UV endonuclease incised cis-syn dimer-containing DNA in a dose-dependent manner and exhibited linear kinetics within that dose range. Enzyme activity was inhibited by the presence of NaCN or NaBH4 with NaBH4 additionally being able to trap a covalent enzyme-substrate product. These last findings confirm that the catalytic mechanism of M. luteus UV endonuclease, like those of other glycosylase/AP lyases, involves an imino intermediate.

Purifications of Micrococcus luteus UV damage-specific or pyrimidine dimer-specific nicking activity have resulted in the isolation of ultraviolet endonuclease proteins with molecular masses ranging from 11 to 18 kDa (2-6). Haseltine et al. (7) proposed that strand scission at a pyrimidine dimer required two activities, an N-glycosylase and an apurinic/apyrimidinic (AP)1 endonuclease. The UV endonuclease-associated N-glycosylase activity would cleave the N-glycosyl bond between the 5′ pyrimidine partner of the dimer and the corresponding deoxyribose moiety. Subsequently, an independent AP endonuclease activity would cleave the sugar-phosphate backbone on the 3′ side of the apyrimidinic sugar moiety. Grafstrom and co-workers (2) modified this proposal by suggesting that the N-glyco-lyase and AP endonuclease activities both reside on the same UV endonuclease molecule. Characterization of an 18-kDa protein has shown that the UV endonuclease prefers thymine-containing dimers over cytosine-containing dimers (under conditions of substrate excess), double-stranded over single-stranded DNA, and apyrimidinic sites at the site of glycosylase action to simple apurinic or apyrimidinic residues (2). The M. luteus UV endonuclease locates pyrimidine dimers, at least in vitro, by a processive sliding mechanism on nontarget DNA (8). The efficiency of this scanning is dependent on both ionic strength and pH. The 3′ terminus generated by UV endonuclease requires further processing by a class II endonuclease before DNA polymerase I and ligase can seal the gap.

The catalytic mechanism of M. luteus UV endonuclease resembles those of a number of other enzymes that perform the initial incision step of base excision repair; bacteriophage T4 endonuclease V (1), Saccharomyces cerevisiae UV endonuclease (9), Escherichia coli endonuclease III and MutY (10, 12), and E. coli Fpg (13, 14) also possess both N-glycosylase and AP lyase activities. Of these functionally related enzymes, our laboratory is most familiar with the reaction mechanism of T4 endonuclease V. T4 endonuclease V employs its NH2-terminal, α-amino group in a nucleophilic attack of the C-1′ sugar carbon of the 5′ nucleotide within the dimer (15, 16). The existence of the resultant imino or Schiff base intermediate is verified experimentally by demonstrating both that cyanide can inhibit the enzyme in a substrate-dependent manner (17, 18) and that NaBH4 can reduce the imino intermediate to a covalent enzyme-substrate product (19). The imino intermediate may or may not undergo a subsequent β-elimination reaction that follows a syn stereochemical course to generate a trans-3′,5′-unsaturated aldehyde and a 5′-phosphate product (20–22). Endonuclease III and Fpg also are believed to utilize primary amino groups to form imino intermediates (23, 24), possibly Lys120 in the case of endonuclease III (25) and a primary amino group at or near the amino terminal in the case of Fpg (24). The AP lyase steps of the M. luteus UV endonuclease (21), endonuclease III (23, 26, 27), and Fpg enzymes are all known to proceed via β-elimina-

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1 The abbreviation used are: AP, apurinic/apyrimidinic; Fpg, 2,6-dihydroxy-5′-formamidopyrimidine (Fapy) DNA glycosylase; PAGE, polyacrylamide gel electrophoresis; FPLC, fast pressure liquid chromatography; bp, base pair(s); SP, sulfyl propyl.
tion. The β-elimination step of endonuclease III, like that of T4 endonuclease V, follows a syn stereochromic course. Thus, a unified catalytic mechanism for the N-glycosylase/AP lyase has emerged; this family of enzymes employs a primary amine nucleophile in its attack on the C-1' sugar carbon of the damaged nucleoside and, in doing so, creates an identifiable imino intermediate (28, 29). Accordingly, the active site residue of the M. luteus UV endonuclease also is hypothesized to be a primary amine.

Since the M. luteus UV endonuclease protein is established in the literature as an 18-kDa protein, it has been compared most frequently with the 16-kDa T4 endonuclease V. This report will demonstrate, however, that the M. luteus enzyme is actually a 31- or 32-kDa protein; a prominent 18-kDa contaminant is MutY, not T4 endonuclease V. In addition to extensive sequence homology with the glycosylase gene revealed that the UV endonuclease shares extensive sequence homology with the E. coli repair proteins endonuclease III and MutY, not T4 endonuclease V. In addition to describing the isolation and cloning of M. luteus UV endonuclease, or more accurately pyrimidine dimer N-glycosylase/AP lyase, we will discuss characterization of the purified enzyme and a preliminary investigation into its reaction mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lyophilized M. luteus cells (ATCC 4698) and lysozyme were obtained from Sigma. The Sephadex G-100, phenyl-Sepharose CL-4B, SP-Sepharose Fast Flow, Mono S, and Mono P matrices or columns were purchased from Pharmacia Biotech Inc.; the Affi-Gel Blue and heparin-agarose matrices were from Bio-Rad. Protein molecular weight markers were purchased individually from Sigma or prestained, low molecular mass markers set from Life Technologies, Inc. were employed. Polyvinylidene difluoride membrane was obtained from Bio-Rad, and microsequencing was carried out in the Protein Sequencing Core Laboratory at Vanderbilt University. [γ-32P]ATP (3,000 Ci/mmol) was purchased from Amer sham. NaCN was obtained from Fisher Scientific Co.

**Isolation of the 18-kDa Ribosomal Protein**

---Based on the amino-terminal sequence of the 18-kDa protein, sequence and antisense 42-mer primers were designed for the protein: 5'-TCGGCCGCCTTCCCCGATCACCATCCGCGG-3' (852) and 5'-GC-GCGCCGGATGATGTATCGGGGCGCCGATGCGGACAT3'- (853). Southern blots performed on restriction digest gels of M. luteus genomic DNA (prepared from the freeze-dried cells) both confirmed that a signal could be detected using either probe and helped to optimize hybridization conditions for the actual cloning procedure. As the initial step in the cloning strategy, a highly skewed bias DNA (d(T)7H2O) was added to the DNA to be cloned. Thepolyvinylidene difluoride paper according to the method of Matsuda (31), excised from the membrane, and microsequenced via Edman degradation.

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McCormick luteus Ultraviolet Endonuclease

LE392 as the host bacterium. Plaques were visible after the bacteria had grown overnight at 37°C, so the plates were chilled for 1 h prior to loading onto a buffer containing 10% (w/v) Bacto-yeast extract, 5% (w/v) NaCl, pH 7.0) medium and allowed to grow for 3 h at 37°C. The cells were lysed by heating the pool, along with the remainder of the Sephadex G-100 pool, was loaded onto a UV-irradiated single-stranded DNA-agarose column (0.5 cm × 30 cm) that had been equilibrated with buffer E. The starting material was batch loaded with buffer containing 200 mM KCl. UV endonuclease activity eluted in 7-ml fractions 16–33 (1050–2,000 mM KCl), 126 ml of a 500-ml linear gradient of 200–4,000 mM buffer B. Five ml of fraction 19 and 6 ml of 20, 21, or 22 were dialyzed separately against 25 mM NaH2PO4, pH 6.8, 100 mM NaCl (buffer E). Each sample was loaded onto an FPLC Mono S column that had been equilibrated with buffer E, and a 38-ml gradient of 100–500 mM NaCl in buffer E was used. Nicking activity eluted consistently in the 260–325 mM NaCl range. Fraction 18 from the UV-irradiated single-stranded DNA-agarose column was dialyzed against 25 mM ethanolamine, pH 10.0, 100 mM NaCl and then injected onto an FPLC Mono P column that had been equilibrated in the same buffer. UV endonuclease activity eluted in 1-ml fractions 3, 4, and 5 of a 38-ml gradient of equilibrium buffer containing 0–8% Pharmacia Polybuffer 96 (pH gradient 10.0–7.0). Mono S 1-ml fractions 17, 19, and 21 from the Affi-Gel Blue fraction 19 run, 15–17 and 19–21 from the Affi-Gel Blue fraction 20 run, 14–19 from the Affi-Gel Blue fraction 21 run, and 16, 17, 19, and 20 from the Affi-Gel Blue fraction 22 run were pooled independently and precipitated with a sixth volume of 70% (w/v) trichloroacetic acid. The samples were subjected to electrophoresis on a 13% SDS-PAGE gel, the 31- and 32-kDa proteins (20–40 pmol each) were transferred to polyvinylidene difluoride membrane, cut into, excised, and microsequenced via Edman degradation.

Cloning of the 31/32-kDa M. luteus UV Endonuclease—Based on the amino acid sequence of the 31- and 32-kDa proteins, the following sense and antisense 38-mer primers were designed for cloning of the UV endonuclease gene: 5'-ATGGAGCAGCGGTTCAAGGGACCCGCAGGGCGGAGAC-3' (1083) and 5'-GTCCTGCCCCGTCGGCGGCTGAGCTCGCTGTCAT-3' (1084). Southern blots were performed on restriction digest gels of M. luteus genomic DNA (prepared from freeze-dried cells) to confirm that a signal could be detected using either probe and to optimize hybridization conditions for the actual cloning procedure. The random PCR products were cloned into a pUC19 vector and sequenced. A 42-bp Pol II library was plated and hybridized at 42°C with 32P-labeled probe 1083. Two of the 10 positive colonies from the initial plating of the M. luteus library survived three subsequent rounds of hybridization. These positives were rescreened as phagedam into a phage vector, and inserts were subcloned into M13mp18, and the M13mp18-clone 3 construct was sequenced. Unfortunately, although a 4-kilobase fragment of genomic DNA had been isolated, only the first 666 bp of the pyrimidine dimer N-glycosylase coding sequence were present. An additional 116-bp fragment was sequenced following the isolation of a larger 5.5-kilobase genomic DNA fragment. Using probes spanning the length of the known 666-bp sequence, a series of restriction digestions and Southern blots was performed to determine the position of the gene of interest within the 5.5-kilobase fragment. A 2.0-kilobase MluI fragment thought to incorporate the full-length gene was isolated and its overhang ends blunt-ended with the SmaI restriction endonuclease.

The pdg gene was sequenced with a battery of sense oligonucleotides and then subcloned into M13mp19 or plasmids for sequencing in the
opposite direction with antisense primers. GC-rich stretches of the sequence often were compressed or poorly resolved, thus making the exact sequence difficult to interpret. Those regions that were recalcitrant to standard Sanger dideoxy sequencing were resequenced with 7-deaza-2′-deoxy-GTP in the deoxynucleoside triphosphate mix or by the Maxam-Gilbert method. Alternatively, sequential and overlapping sections of the gene were amplified by polymerase chain reaction and then sequenced individually.

Purification Scheme 3:

Twenty-five g of M. luteus cells was suspended in 1,250 ml of buffer B minus ethylene glycol, allowed to hydrate overnight, and then lysed for the next 24 h by the addition of 400 μg/ml lysozyme into the cell slurry. The KCl concentration of the slurry was raised to 300 mM immediately by precipitation. Cellular debris was pelleted by 30-min rounds of centrifugation at 8,000 × g, and the resultant supernatant was loaded onto a single-stranded DNA-agarose column (20 cm² × 75 cm) that had been equilibrated in buffer B. A 400-ml linear gradient of 1.8–0 M NaH₂PO₄, pH 6.8, 10% ethylene glycol (buffer H) was run, but no nicking activity was found until NaH₂PO₄, pH 6.8, 1 mM EDTA, 100 mM KCl, 10% NaH₂PO₄, pH 6.8, 1 mM EDTA (buffer F), and applied to an SP-Sepharose column (1.8 cm² × 45 cm) that had been equilibrated in buffer F. A 500-ml linear gradient of 0–800 mM KCl in buffer F concentrated enzyme activity in 3.5-ml fractions 24–36 (5–70 mM KCl, 45 cm) that had been equilibrated with 25 mM NaH₂PO₄, pH 6.8, 1 mM EDTA, 100 mM KCl, 10% NaH₂PO₄, pH 6.8, 1 mM EDTA (buffer F). Micrococcus luteus Ultraviolet Endonuclease Damage- and Mismatch-specific Nicking Activity on Plasmid DNA

In purification schemes 1–3, fractions were monitored routinely for their ability to nick irradiated versus unirradiated plasmid DNA. pBR322 plasmid DNA was irradiated with 254-nm light at 100 micro-watts/cm² for 245 s to generate 20–25 pyrimidine dimers/plasmid mol-electron (32). The DNA was then diluted from approximately 1 μg/ml to 0.100 μg/ml in 25 mM NaH₂PO₄, pH 7.5, 1 mM EDTA, 100 mM ethylene glycol serum albumin (puriﬁcation schemes 1 and 2) or to 0.075 μg/ml in 25 mM NaH₂PO₄, pH 6.8, 25 mM NaCl, 1 mM EDTA, 100 mM ethylene glycol serum albumin (puriﬁcation scheme 3). Varying amounts of the column fractions, usually 1–5 μl or dilutions thereof, were incubated with 20 μl of irradiated or unirradiated pBR322 (2.0 μg of DNA in purification scheme 1; 4.0 μg of DNA in puriﬁcation scheme 2; or 30 μg in purification scheme 3) for 30 min at 37°C. Reactions were terminated by the addition of an equal volume of electrophoresis loading buffer (50 μl Tris-HCl, pH 8.0, 20 mM EDTA, 40% (w/v) sucrose, 2% (w/v) SDS, 0.02% (w/v) bromphenol blue, 0.02% (w/v) xylene cyanol). Form I (supercoiled), form II (nicked circular), and form III (linear) DNAs were resolved by electrophoresis through a 1% (w/v) agarose gel in 40 μl Tris-GAC, pH 8.0, 1 mM EDTA running buffer. The gel was stained in 0.5 μg/ml ethidium bromide so that the three topological forms of pBR322 could be visualized on a longwave UV lightbox. To quantitate the data, images of the gels were captured by a digital camera system (The Imager, Appligene) and then analyzed using Bio-Image software (Millipore).

M. luteus UV Endonuclease Damage and Mismatch-specific Nicking Activities on Site-speciﬁc Oligonucleotide Duplexes

In puriﬁcation scheme 3, fractions were monitored for both their ability to nick irradiated plasmid DNA and their ability to nick a 49-bp oligonucleotide duplex containing a site-speciﬁc cis-syn thymine dimer.

In addition, puriﬁed M. luteus UV endonuclease (phenyl-Septaphorase fraction 315) was tested for its ability to nick 49-mers containing a trans-syn-1, (6–4), or Dewar thymine dimer (33), a 37-mer containing a 5,6-dihydrouracil lesion, and 50-mers containing an A·G or A·C mismatch. The trans-syn-1, (6–4), and Dewar thymine dimer-containing duplexes shared the following sequence in the damaged strand: 5′- AGCTACCTGCTGCAGAACCGCATGCTAATGCGAATT-3′. The cis-syn damaged strand had a slightly altered sequence: 5′- AGCTACCTGCGCAATGCTAAATGCGAATT-3′. Each dimer type bridged the two thymines in boldfaced positions 21 and 22 of the 49-mer labeled on its 5′ end with T4 polynucleotide kinase and then annealed to its complement. Depending upon the experiment, varying amounts of phenyl-Septaphorase fraction 315 or control enzyme were incubated at 37°C for the indicated length of time with the appropriate duplex 49-mer (0.4 ng = 12.5 fmol in a 10-μl volume). Each type of loading buffer was 37-mer duplex (0.8 ng = 32 fmol in a 10-μl volume), 50-mer duplex (0.5 ng = 16 fmol in a 10-μl volume) in 25 mM NaH₂PO₄, pH 6.8, 1 mM EDTA, 100 μg/ml bovine serum albumin. Reactions were terminated either by placing the reaction tubes in a dry ice-ETOH bath or by treating the reaction mixtures with 1 μl piperidine for 30 min at 85°C. When piperidine treatment was carried out, reaction samples were dried down in a Savant SpeedVac and then resuspended in twice their original reaction volume of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromphenol blue, 0.02% (w/v) xylene cyanol). When piperidine treatment was not carried out, an equal volume of formamide loading buffer was added to the samples. All samples were heated for 3–5 min at 80°C prior to being loaded onto 15% polyacrylamide gels containing 8 M urea. The oligonucleotide bands were visualized by autoradiography of the wet gels with Hyperfilm-MP film (Amersham Corp.) at ~70°C, typically for several hours with two DuPont Quanta III intensifying screens enclosed in the cassette. When it was necessary to obtain quantitative results, the wet gels were also scanned on a Phosphorimager 450 machine (Molecular Dynamics) and the data analyzed using Image Quant software (Molecular Dynamics).

RESULTS

Puriﬁcation and Cloning of the 18-kDa Ribosomal Protein—The original intent of puriﬁcation scheme 1 was to isolate the 18-kDa M. luteus UV endonuclease that had been reported in the literature (2). Since the M. luteus UV endonuclease was believed to be similar in structure to T4 endonuclease V, the puriﬁcation scheme incorporated many of the chromatographic steps that are routinely employed by our laboratory to purify endonuclease V: single-stranded DNA-agarose, Sephadex G-100, heparin-agarose, phenyl-Sepharose, and FPLC Mono S (34). Column fractions were assayed for M. luteus UV endonuclease activity by performing nicking assays on UV-irradiated versus nondamaged pBR322 plasmid DNA. The protein makeup of the fractions was assessed via SDS-PAGE coupled to either Coomassie Blue or silver staining. After following the UV damage-speciﬁc nicking activity of the M. luteus lysate over ﬁve different column types, with some columns being run twice, an 18-kDa protein emerged as the most prominent band. Contaminants were still present even after the FPLC Mono S runs, however, with a 31/32-kDa doublet being particularly noticeable (Fig. 1A). Consequently, the Mono S fractions were pooled according to protein content, and the 18-kDa protein was puriﬁed further to ~80% homogeneity on a second phenyl-Septaphorose column. Microsequencing of the ﬁrst 14 amino acids of the 18-kDa protein (Table I) and knowledge of the unique codon usage pattern of M. luteus (35, 36) enabled us to design sense and antisense 42-mer primers to clone the potential endonuclease gene.
Purification and Cloning of the 31/32-kDa M. luteus UV Endonuclease—After concluding that the 18-kDa protein was not the M. luteus UV endonuclease, the protein and activity profiles collected during purification scheme 1 were reexamined. Reinspection of the data showed that the peaks for the 18-kDa protein and UV damage-specific nicking activity had been slightly offset; faint 31- and 32-kDa protein bands correlated more directly with protein activity. Purification scheme 2 therefore was undertaken with the purpose of isolating this protein doublet. It included the following chromatographic steps: single-stranded DNA-agarose, Affi-Gel Blue, phenyl-Sepharose, Sephadex G-100, heparin-agarose, UV-irradiated single-stranded DNA-agarose, FPLC Mono S, and FPLC Mono P. As was expected from the observations described above, the UV damage-specific nicking activity was retained even after the removal of small molecular weight proteins (M, 11,000–18,000) from the enzyme preparation. Fig. 1, B and C, illustrates the correspondence between the amount of 31/32-kDa protein and the level of UV damage-specific nicking activity for several FPLC Mono P fractions. Quantitative analysis of Fig. 1B indicated that lane 1 (fraction 3) contained 5.6 times as much protein at the 31- and 32-kDa positions as did lane 2 (fraction 4), but no protein was detected in lane 3 (fraction 5).

Micrococcus luteus Ultraviolet Endonuclease

TABLE I

| Protein band | NH₂-terminal amino acid sequence |
|--------------|----------------------------------|
| 18-kDa       | (M) S R I G R L P I T I P A G     |
| (scheme #1)  |                                  |
| 31-kDa       | M E TEST GTPTGETRLALVRRA         |
| (scheme #2)  |                                  |
| 32-kDa       | M E TEST GTPTGETRLALVRRARRI      |

Cloning identified an 804-bp open reading frame encoding a protein of 268 amino acids with a calculated molecular mass of 29,306 Da (Fig. 2A). Curiously, a second stop codon was present at bp position 838 which could potentially signal the termination of a longer protein of 279 amino acids with a calculated molecular mass of 30,340 Da. Although we were unsure of how the M. luteus transcription machinery might bypass the first OPA codon, perhaps through an amber suppressor-type mechanism, it seemed plausible that the 268- and 279-residue proteins corresponded to the two forms of purified UV endonuclease protein with apparent molecular masses of 31 and 32 kDa, respectively. Indeed, amino-terminal sequence data obtained for the first 35 residues of the 31-kDa protein and the first 24 residues of the 32-kDa protein matched the NH₂-terminal sequence predicted by the cloned gene. Comparable to other M. luteus genes that have been sequenced, pdg possessed an overall GC content of 72%, and 94% of its codons contained a G or C in the third position. Also, a purine-rich, Shine-Dalgarno-like
sequence was present 14 to 8 bases upstream of the start codon.

A GenBank search revealed that the protein deduced from the pdg open reading frame shared significant homology with two E. coli repair proteins, endonuclease III (31% identity across the length of the M. luteus protein) and MutY (22% identity) (Fig. 2B), and an uncharacterized, PfV1 plasmid-encoded protein from Micrococcus luteus (the open reading frame 10 product, 16% identity) (25, 39). Across all four proteins, the regions surrounding the endonuclease III thymine glycol binding site (Ala-113 through Arg-119) and the putative [4Fe-4S]2+ cluster motif (C-X6-C-X2-C-X4-C) were particularly well conserved. The nucleotide sequence for the M. luteus pdg gene has been submitted to GenBank and assigned accession number U22181.

Purification scheme 3 accomplished two goals: (i) a more streamlined purification procedure was developed, and (ii) enough protein was isolated to characterize the enzyme. The 32-kDa partner of the 31/32-kDa UV endonuclease pair was purified to apparent homogeneity after three chromatographic steps: single-stranded DNA-agarose, SP-Sepharose, and phenyl-Sepharose. Active 31-kDa proteins were eluted off the phenyl-Sepharose column prior to its 32-kDa partner, but it was impure (data not shown). Pure 32-kDa protein was eluted off the phenyl-Sepharose column in fractions 300–325 only after extensive, postgradient washing with low salt buffer (Fig. 3A). Consequently, these fractions were very dilute; each silver stain band represented 400 μl of trichloroacetic acid-precipitated material. Each fraction was capable of nicking a 49-mer oligonucleotide duplex containing a site-specific cis-syn cylobutane thymine dimer. The resultant 20-mers comigrated with the β-elimination product generated by T4 endonuclease V (Fig. 3B, lane 18). Fraction 330 lacked the 32-kDa protein and was incapable of nicking the 49-mer substrate.

Characterization of the M. luteus UV Endonuclease—The recent synthesis by Smith and Taylor (33) of a set of deoxyligonucleotide 49-mers containing defined thymidylyl-(3′ → 5′)-thymidine photoproducts allowed us to investigate the dimer specificity of purified UV endonuclease. The 32-kDa protein (phenyl-Sepharose fraction 315 from purification scheme 3) incised duplex 49-mer cis-syn but not trans-syn-1, (6–4), or Dewar thymine dimers (Fig. 4A). Nor could the UV endonuclease nick a duplex 37-mer containing a 5,6-dihydrouracil-A:C mismatch, known substrates of MutY (Fig. 4, B and C) (41). Although it is not evident from the exposure presented in Fig. 4C, MutY was able to cleave the A:C substrate at 4% of the rate that it cleaved the A:G substrate. UV endonuclease product PhosphorImager counts were slightly elevated for the 5,6-dihydrouracil and A:G substrates relative to their undamaged counterparts, but, at most, the counts approached 2% of the positive control counts or <1% of total counts. Considering that piperidine treatment was not required to detect the cleavage product of the cis-syn thymine dimer-containing substrate and that more UV endonuclease enzyme was introduced into the 5,6-dihydrouracil-A:C mismatch assays than into the pyrimidine dimer assays, its cis-syn pyrimidine dimer-specific nicking capability probably represents the biologically relevant role of M. luteus UV endonuclease. The purified protein nicked cis-syn dimer-containing DNA in a concentration-dependent manner, and piperidine treatment did not enhance the conversion of 49-mer to 20-mer product (Fig. 5). Piperidine treatment did increase the mobility of the 20-mer reaction product, however; removal of the 3′ sugar fragment through β-elimination left a 3′ phosphate terminus with a faster mobility than the original aldehyde (data not shown). The fact that piperidine treatment failed to convert

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**Fig. 2. DNA sequence of the pdg gene and amino acid sequence alignment of UV endonuclease, endonuclease III, and MutY.** Panel A presents the nucleotide sequence of the pdg open reading frame and its flanking 5′ and 3′ sequences. The deduced amino acid sequence of the pyrimidine dimer N-glycosylase/AP lyase protein is given above the DNA sequence. The initiation and termination codons are shown in uppercase. MutY DNA repair proteins. Regions of strong homology are indicated by a consensus sequence with the conserved C-X6-C-X2-C-X4-C coordination motif and the putative catalytic residues for endonuclease III and UV endonuclease (Lys-120 and Lys-135, respectively) are highlighted with boldfaced type.
Samples were then subjected to electrophoresis on a 15% polyacrylamide gel containing 8 M urea for 60 min at 37°C before they were terminated by the addition of formamide loading buffer. The autoradiographic results of an accompanying nicking assay are given in UV endonuclease.

Lane M contained oligonucleotide sizing markers ranging from 8 to 32 bases. 32P-Labeled 49-mer substrate and 20-mer product were visualized via autoradiography of the wet gel. The complexes migrated more slowly than the 49-mer substrate alone and were located just beneath the wells.

Evidence for an Imino Intermediate in the M. luteus UV Endonuclease Reaction—The catalytic mechanism of the M. luteus UV endonuclease was hypothesized to proceed via an imino intermediate like that of other N-glycosylase/AP lyases. Such an intermediate was detected both indirectly by demonstrating that cyanide inhibited the enzyme and directly by trapping the intermediate as (a) covalent enzyme-substrate product(s) with the reducing agent NaBH₄. UV endonuclease was reacted with cis-syn dimer-containing duplex 49-mer in the presence of NaCl or equimolar concentrations of NaCN (Fig. 7, A and B). Fifty percent inhibition of the reaction occurred around 6 mM NaCN, an IC₅₀ very similar to the 3–5 mM range observed for T4 endonuclease V (19). Cyanide, unlike NaBH₄, reacts with an imino intermediate to form a slowly reversible tetrahedral complex that cannot be isolated by denaturing PAGE. Therefore, no shifted bands representing enzyme-substrate complexes were seen in the Fig. 7A autoradiograph. Stable complexes were formed when NaBH₄ was present in the reaction at ≥10 mM (Fig. 7C). The complexes migrated more slowly than the 49-mer substrate alone and were located just beneath the wells. It is not understood how the upper and lower complexes differed, but they were formed somewhat quantitatively according to the amount of enzyme added to the reactions. The PhosphorImager covalent complex counts in Fig. 7C, lane 14, for instance, totalled 13,885, almost four times the 3,864 counts totalled in lane 7, and translated into approximately 1% of the substrate molecules being cross-linked to the

A significant number of AP sites to single-stranded breaks suggested that the AP lyase activity of the M. luteus UV endonuclease was at least as strong as its N-glycosylase activity. Had the UV endonuclease possessed a weak AP lyase, AP sites would have remained in the DNA following the N-glycosylase cut. Piperidine treatment would have created DNA breaks, and an increase in 20-mer product would have been observed. Since the dose dependence of the UV endonuclease was not perfectly linear, i.e. 2.50 μl of enzyme converted 100% of the 49-mer to 20-mer, whereas 1.25 μl of the enzyme converted only 30% of the substrate to product, the kinetics of the UV endonuclease were examined (Fig. 6). As would be expected for a situation of substrate excess or even for this situation of approximately equimolar substrate and enzyme concentrations, initial reaction velocities were proportional to the enzyme concentration:

\[
\text{Initial velocity} = k \cdot [\text{enzyme}] 
\]

where \(k\) is the rate constant.

The UV endonuclease also shows a dose-dependent increase in 20-mer product. This increase is due to the time-dependent nature of the reaction, as well as due to the fact that the enzyme has a higher affinity for the 37-mer than for the 21-mer. The dose dependence of the UV endonuclease was at least as strong as its N-glycosylase activity.
The newest member of an emerging family of [4Fe-4S]2+ cluster DNA repair glycosylases is the M. luteus UV endonuclease. Increasing amounts of phenyl-Sepharose fraction 315 (2.5 μl and 2, 4, 8, 16, 32, 64, 128, 256, and 512 × dilutions thereof) were incubated with cis-syn thymine dimer-containing duplex 49-mer (0.4 ng - 12.5 fmol) for 60 min at 37°C. Reactions were terminated either by placing the reaction tubes in a dry ice-EtOH bath (C) or by heating the mixtures with 1 μl piperidine for 30 min at 85°C (D), a treatment that served to convert any remaining AP sites to single-stranded breaks. After the addition of formamide loading buffer, samples were subjected to electrophoresis on a 15% polyacrylamide gel containing 8 M urea. 32P-Labeled 49-mer substrate and 20-mer product were visualized by both autoradiography and PhosphorImaging of the wet gel. The relative amounts of substrate remaining and product generated were determined using Image Quant software.

For a number of years, our laboratory tried unsuccessfully to clone the M. luteus UV endonuclease using strategies designed to exploit the suspected homology between the M. luteus enzyme and T4 endonuclease V (42). As a final strategy, we chose to sequence its amino terminus, and design best guess oligonucleotide probes to screen a randomly sheared genomic ZAP II library. Contrary to what we had expected, the M. luteus UV endonuclease turned out to be a 31/32-kDa protein, not a low molecular weight protein in the range of 11,000–18,000. Furthermore, the product of the M. luteus pdg gene resembled the E. coli repair proteins, endonuclease III and MutY, not endonuclease V.

The M. luteus UV endonuclease is intriguing in that it constitutes the newest member of an emerging family of [4Fe-4S]2+ cluster DNA repair glycosylases. Moreover, these glycosylases possess widely divergent substrate specificities. The M. luteus UV endonuclease cleaves DNA at pyrimidine dimers, endonuclease III releases thymine glycols and a number of other ring-saturated and ring-fragmented derivatives of thymine (25), and MutY removes undamaged adenines that are mispaired with 8-oxoguanine lesions, 8-oxoadenine lesions, guanines, or cytosines (41, 43). The function of the M. thermoformicicum protein has not yet been determined, but it has been speculated to be involved in the repair of G:T mismatches that result from the deamination of 5-methylcytosine by another pFV1 plasmid-encoded protein, the GGCC-recognizing methyltransferase (39). Thus, the [4Fe-4S]2+ cluster glycosylases either share a common core structure onto which base-specific recognition motifs have been added or recognize the distortion introduced by DNA damage as opposed to the damage itself. At least in endonuclease III, the [4Fe-4S]2+ cluster appears to contribute to the structural integrity of the protein rather than to play a direct role in catalysis (44, 45). One additional piece of evidence supports our hypothesis that the UV endonuclease and endonuclease III are structurally similar: the SP-Sepharose Fast Flow resin served as an invaluable tool in the purifications of both enzymes (46). To eliminate any possibility that we had accidentally cloned the M. luteus homolog of either endonuclease III or MutY, we demonstrated that purified protein was incapable of cleaving a 5,6-dihydrouracil-containing substrate or mismatch substrates, respectively.
apparent specific activity of the purified UV endonuclease was comparable to that of T4 endonuclease V, but the M. luteus UV endonuclease possessed an AP lyase activity equal to its N-glycosylase activity. Its propensity not to dissociate prior to the substrate complex was trapped more readily with NaBH₄ than the T4 endonuclease V imino intermediate. Other investigators have reported that the N-glycosylase activity of the M. luteus UV endonuclease is up to an order of magnitude greater than its apparent AP lyase activity, even in partially purified preparations that may be contaminated by multiple AP endonucleases (2, 7, 8). These findings are difficult to reconcile with our own unless, as noted by Hamilton and Lloyd (8), the AP lyase activity is more labile over time than is the N-glycosylase activity. Finally, high salt concentrations (100 mM salt on top of 25 mM NaH₂PO₄, pH 6.8 buffer) reduced the nicking activity of the UV endonuclease. One of two explanations seems likely: (i) either salt-sensitive processivity facilitated the cleavage of the substrate even though it was only 49 bp long, or (ii) salt inhibited the UV endonuclease via a mechanism that was independent of processivity. The protein was clearly distinct from the M. luteus ~35-kDa class II AP endonucleases A and B in that they require Mg²⁺ and are sensitive to inactivation by EDTA (47, 48).

The catalytic mechanism of the M. luteus UV endonuclease has been shown to involve a Schiff base intermediate. This finding strengthens our theory that all N-glycosylase/AP lyases proceed via an imino intermediate (28, 29). Lys-120, a basic residue, was implicated recently in the reaction mechanism of endonuclease III; mutagenesis of Lys-120 to Gln-120 resulted in a 10³-fold decrease in the enzyme's activity compared with wild type (25). An analogous Lys-135 residue is present in the enzyme-substrate complex(es) visualized both by autoradiography and PhosphorImager/Image Quant data. The inset arrows point at enzyme-substrate complexes. As is indicated by the slash marks above the wells in panels A and C, the samples spread outward as they ran through the polyurea denaturing gels; the residue that can be seen in each well will not align perfectly with the 49-mer or 20-mer bands.

FIG. 7. Inhibition by NaCN or NaBH₄ of M. luteus UV endonuclease nicking of cis-syn thymine dimer-containing oligonucleotide duplex. Panel A, buffer (4.0 μl, lanes 1 and 2) or phenyl-Sepharose fraction 315 (4.0 μl, lanes 3–12) was incubated with control (lanes 1 and 3) or cis-syn thymine dimer-containing (lanes 2 and 4–12) duplex 49-mer (0.8 ng = 25 fmol). NaCl (odd lanes) or NaCN (even lanes) was present in the reaction mixtures at 0 mM (lanes 1–4), 5 mM (lanes 7 and 8), 10 mM (lanes 9 and 10), or 25 mM (lanes 11 and 12). Panel B, phenyl-Sepharose fraction 315 (4.0 or 16.0 μl, lanes 1–7 or 8–14) or buffer (16.0 μl, lane 15) was incubated with cis-syn dimer-containing duplex 49-mer (0.8 ng = 25 fmol). NaCl or NaBH₄ was present in the reaction mixtures at 0 mM (lanes 1, 8, and 15), 5 mM (lanes 2, 3, 9, and 10), 10 mM (lanes 4, 5, 11, and 12), or 100 mM (lanes 6, 7, 13, and 14). All reactions were terminated after 60 min at 37 °C by the addition of formamide loading buffer, and the samples were subjected to electrophoresis on 15% polyacrylamide gels containing 8 M urea. M lanes contained oligonucleotide sizing markers ranging from 8 to 32 bases. ³²P-Labeled 49-mer substrate, 20-mer product, and enzyme substrate complex(es) were visualized both by autoradiography and PhosphorImaging of the wet gels. Panels A and C show the autoradiographic results of these experiments; panels B and D summarize the PhosphorImager/Image Quant data. The inset in panel C superimposes a 1-week exposure of the gel over a 4-h autoradiograph. Although its placement is essentially accurate, the inset was shifted downward just slightly so as to not obscure the residues marking the wells. The inset arrows point at enzyme-substrate complexes. As is indicated by the slash marks above the wells in panels A and C, the samples spread outward as they ran through the polyurea denaturing gels; the residue that can be seen in each well will not align perfectly with the 49-mer or 20-mer bands.
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