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The Catalytic Mechanism of a Pyrimidine Dimer-specific Glycosylase (pdg)/Abasic Lyase, chlorella virus-pdg*

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The repair of UV light-induced cyclobutane pyrimidine dimers can proceed via the base excision repair pathway, in which the initial step is catalyzed by DNA glycosylase/abasic (AP) lyases. The prototypical enzyme studied for this pathway is endonuclease V from the bacteriophage T4 (T4 bacteriophage pyrimidine dimer glycosylase (T4-pdg)). The first homologue for T4-pdg has been found in a strain of *Chlorella* virus (strain *Paramecium bursaria Chlorella* virus-1), which contains a gene that predicts an amino acid sequence homology of 41% with T4-pdg. Because both the structure and critical catalytic residues are known for T4-pdg, homology modeling of the *Chlorella* virus pyrimidine dimer glycosylase (cv-pdg) predicted that a conserved glutamic acid residue (Glu-23) would be important for catalysis at pyrimidine dimers and abasic sites. Site-directed mutations were constructed at Glu-23 to assess the necessity of a negatively charged residue at that position (Gln-23) and the importance of the length of the negatively charged side chain (Asp-23). E23Q lost glycosylase activity completely but retained low levels of AP lyase activity. In contrast, E23D retained near wild type glycosylase and AP lyase activities on *cis-syn* dimers but completely lost its activity on the *trans-syn* II dimer, which is very efficiently cleaved by the wild type cv-pdg. As has been shown for other glyscosylases, the wild type cv-pdg catalyzes the cleavage at dimers or AP sites via formation of an imino intermediate, as evidenced by the ability of the enzyme to be covalently trapped on substrate DNA when the reactions are carried out in the presence of a strong reducing agent; in contrast, E23D was very poorly trapped on *cis-syn* dimers but was readily trapped on DNA containing AP sites. It is proposed that Glu-23 protonates the sugar ring, so that the imino intermediate can be formed.

UV light damages DNA through the formation of two types of pyrimidine dimers: cyclobutane pyrimidine dimers and 6-4 photoproducts (1). One mechanism for the repair of the cyclobutane pyrimidine dimer is the base excision repair pathway, which is initiated by a DNA glycosylase/abasic (AP)[1] lyase. Although many DNA-containing viruses that have sustained UV-induced DNA damage, use host cell enzymes to repair their DNA, the bacteriophage T4 is unusual in that it encodes an enzyme, endonuclease V (T4-pdg, pyrimidine dimer glycosylase), that cleaves the N-glycosyl bond of the 5′ thymine of the dimer and then subsequently cleaves the phosphodiester backbone, producing a ring opened sugar as an α, β unsaturated aldehyde (2–4). T4-pdg has been characterized extensively since its discovery over 40 years ago, and its structure and mechanism of catalysis have been recently reviewed (5, 6).

The first eukaryotic homologue of T4-pdg has been found to be encoded within the genome of an algal virus, *Paramecium bursaria Chlorella* virus-1. As a prelude to investigating structure-function relationships in *Chlorella* virus pyrimidine dimer glycosylase (cv-pdg), it is advantageous to utilize molecular modeling tools to direct biochemical analyses. However, in order to justify molecular modeling studies of a protein of which the structure has not been determined by x-ray crystallography or NMR spectroscopy, it is essential that the reference enzyme (T4-pdg): 1) be highly homologous to the protein of interest (cv-pdg), 2) has had its crystal structure solved as both the apoenzyme and as a complex with substrate containing DNA, 3) has had the critical active site residues established, and 4) recognize the same substrate and catalyze a similar reaction mechanism. In this study, T4-pdg is ideally characterized to serve as a homologous reference map for the study of cv-pdg, for the following reasons. First, the gene encoding cv-pdg predicts a protein that has a 41% identity with T4-pdg (7). Additionally, homologous genes from over 40 *Chlorella* virus genomes reveal a very high degree of sequence conservation.[2] Second, the crystal structure (9) and the co-crystal structure of T4-pdg with cyclobutane pyrimidine dimer-containing DNA (10) have been solved at high resolution. Third, two key residues have been identified in T4-pdg to catalyze the combined glycosylase/AP lyase activity, in which the α-amino group of Thr-2 and Glu-23 act in concert to catalyze the nucleophilic displacement reaction (11–17). Fourth, as originally hypothesized for T4-pdg, cv-pdg has been hypothesized to initiate repair by protonation on the damaged base followed by glycosidic bond destabilization. This is followed by a nucleophilic attack at the C1′ of the deoxyribose sugar via a primary amine present in the protein (18). Similar reaction mechanisms have been described for other enzymes in the base excision repair pathway: formaminopyrimidine DNA glycosylase (19, 20), MutY (21), and the human homologue of endonuclease III (NTH1) (22).

Additionally, the initial characterization of the properties of cv-pdg revealed that despite its high degree of sequence similarity with T4-pdg, there were significant differences in the activities on different isomers of the cyclobutane pyrimidine dimer (23). In contrast to T4-pdg, cv-pdg was able to efficiently

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[1] The abbreviations used are: AP, abasic; T4-pdg, T4 bacteriophage pyrimidine dimer glycosylase; cv-pdg, *Chlorella* virus pyrimidine dimer glycosylase; amp, ampicillin; PCR, polymerase chain reaction.

[2] J. Van Etten, personal communication.

cleave the *trans-syn* II photoisomer, possibly suggesting a subtly different active site or binding pocket.

Thus, with all of these available data, modeling studies and electrostatic potential mapping have been carried out to predict the structural similarities between T4-pdg and cv-pdg (23). In this study, we investigated the catalytic mechanism of cv-pdg by using homology modeling to design site-directed mutations to test the role of Glu-23 in both the glycosylase and AP lyase reactions on thymine dimers and AP sites.

EXPERIMENTAL PROCEDURES

Cloning of Chlorella Virus Pyrimidine Dimer Glycosylase—The cv-*pdg* gene (A50L) was PCR-amplified from the pUC19 vector (7) to generate an *Nde*I site on the 5′-end and a *Hin*dIII site on the 3′ side. The PCR primers were 5′-CATATGACACGTGTGAATCTCGTACCGG-3′ and 5′-AAGCTTAATTATTGCTGGTTTTAGCTTTCGTG-3′. This fragment was subcloned into the pET-lla vector (Stratagene) by the NIEHS Molecular Biology Core at the University of Texas Medical Branch under the supervision of Dr. Thomas G. Wood. PCR conditions can be obtained upon request.

Homology Model of cv-pdg Based on the Endonuclease V Structure—The amino acid sequence of T4-pdg was aligned with the predicted amino acid sequence of the cv-pdg protein using the Look computer modeling package SegMod (Molecular Applications Group, Palo Alto, CA). The SegMod algorithm can be briefly described as follows. The cv-pdg sequence was divided into short segments, and these segments were matched to fragments in the Brookhaven Protein Data Bank structural data base. The fragments were combined into a framework based on a template structure, in this case, the known structure of T4-pdg (10). Multiple structures of the model were built and averaged into a final structure, which was then stereochemically refined using 500 rounds of energy minimization. The RMS deviation of backbone atoms relative to the T4-pdg structure was 1.6 Å.

The coordinates of the modeled cv-pdg structure were used to calculate a relative electrostatic potential map using GRASP (24). The GRASP Poisson-Boltzmann calculation used the following parameters: interior dielectric constant, 2.0; exterior dielectric constant, 80; water probe radius, 2.0; and salt concentration, 0.0. The relative potential values were mapped onto the Connolly surface of the model. The same procedure was followed with the T4-pdg structure for comparison with the modeled cv-pdg protein.

Oligonucleotide Site-directed Mutagenesis of cv-pdg—Site-directed mutagenesis was carried out on the pET-11a vector containing the cv-*pdg* gene using the Quikchange mutagenesis kit (Stratagene). The sequence of the primers used to carry out the mutagenesis of the cv-*pdg* gene were as follows: 5′-GCTTCGGAATCATCTTAAGGTCACGAAATTCTGCCATGAG-3′ (E23D cv-pdg), 5′-CTCATGGCAGAATTTCGTGACCTTAAGATGATTCCGAAGGC-3′ (E23D cv-pdg), 5′-CTTCGGAATCATCTTAAGTTGACGAAATTCTGCCATGAGAT-3′(E23Q cv-pdg), and 5′-ATCTCATGGCAGAATTTCGTCAACTTAAGATGATTCCGAAG-3′ (E23Q cv-pdg), where the mismatched nucleotides are underlined. PCR conditions are available upon request. Following the PCRs, the templates were digested with *Dpn*I, and the PCR product was visualized following separation on a 0.8% agarose gel and staining with ethidium bromide. An aliquot of the PCR (4 μl) was used for transformation into Supercompetent XL1 Blue bacterial cells (Stratagene). After 1 h at 0 °C, the transformation reaction was heat-shocked for 1 min at 42 °C. The cells were allowed to recover for 1 h at 37 °C in 2X-YT medium (pH 7.0) (16 g of bacto-tryptone, 10 g of bacto-yeast extract, 5 g of NaCl per liter of deionized water). Cells were then plated out on LB plates that contained 100 μg/ml ampicillin (amp). Plasmid preparations were prepared from amp resistant colonies and analyzed by automated sequencing (NIEHS Molecular Biology Core Facility) to confirm each mutation.

Purification of Chlorella Virus Pyrimidine Dimer Glycosylase Mutants E23D and E23Q—The purification scheme for the mutant enzymes differed slightly from that previously described for the wild type enzyme (7). After sequencing each mutated gene to ensure that the only mutation generated was the mutation of interest, the plasmid was introduced into *E. coli* BL21DE3, which is used for expression from this plasmid. This cell is a DE3 lysogen in which the gene for the T7 RNA polymerase has been inserted in the λ int gene, such that the lysogen remains stable. The T7 RNA polymerase gene is induced by induction with isopropyl-1-thio-β-D-galactopyranoside. Cultures (2 liters) were grown in LB medium containing 100 μg/ml amp at 30 °C for 6 h. Cells were pelleted by centrifugation at 4000 × *g* for 15 min at 4 °C and resuspended in Buffer A (50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5% glycerol, 5 mM dithiothreitol, 100 mM NaCl, and 0.6 M sucrose). The cells were broken using a French press at a constant pressure of 9000 p.s.i. The lysates were cleared of cellular debris by centrifugation at 8000 × *g* for 30 min at 4 °C. The cleared lysates were loaded onto two 60-ml tandem Q-Sepharose-SP-Sepharose columns that had been previously equilibrated with Buffer B (25 mM sodium phosphate (pH 7.5), 1 mM EDTA, 0.5 mM dithiothreitol, and 100 mM NaCl). The mutant cv-pdg proteins flowed through the Q-Sepharose but bound to the SP-Sepharose matrix and were eluted with a linear gradient of Buffer B from 0.1 M to 1.0 M NaCl (200 ml total volume). Fractions were collected and monitored by Western blot analysis and Coomassie Brilliant Blue R-250 staining of 15% polyacrylamide-SDS gels. The fractions that contained the mutant cv-pdg proteins were pooled, dialyzed, and loaded onto a 25-ml single-stranded DNA cellulose column that had been equilibrated with Buffer B. A linear gradient was run over the column of Buffer B from 0.1 M to 1.0 M NaCl (150 ml total volume). Several fractions appeared to contain pure cv-pdg by Coomassie Brilliant Blue staining and were subsequently analyzed via silver staining.

Qualitative in Vivo Assay of Mutant Enzyme Activity—E. coli strain AB2480 (uvrA, *recA*$^-$) containing the pET-11a vector with either wild type cv-pdg, E23D cv-pdg, or E23Q cv-pdg was grown overnight at 37 °C in LB medium containing 100 μg/ml amp. Aliquots of these cultures were streaked onto an LB agar plate that also contained 100 μg/ml amp. The liquid bacterial medium was allowed to dry, and areas on the plate were irradiated with 254-nm light at 1.0 μW/cm^2 for increasing amounts of time. Following irradiation, the plates were incubated in the dark at 37 °C for 12 h.

Gel Mobility Shift Binding Assay—The *cis-syn* 49-mer, *trans-syn II* 49-mer, reduced AP 49-mer, pyrrolidine (P) 25-mer (5′-GGATAGTGTCCAPGTTACTCGAAGC-3′), and tetrahydrofuran (F) 25-mer (5′-GGATAGTGTCCAFGTTACTCGAAGC-3′) were 5′-end-labeled with [γ-^{32}P]ATP and annealed to the appropriate complementary oligonucleotide with an adenine opposite the damaged nucleotide. The reduced AP 49-mer was generated by the same method as the AP 49-mer substrate except that subsequent to treatment with UDG, the DNA was treated with 100 mM $NaBH_4$ for 10 min at 25 °C. Binding of the E23D cv-pdg or E23Q cv-pdg was assessed by gel mobility shift analysis. The reactions were conducted in a 20-μl total volume with the appropriate dilutions of the enzymes, and 20 pM substrate DNA duplexes in 25 mM NaH_2PO_4 (pH 6.8), 100 mM NaCl, and 100 μg/ml bovine serum albumin and a 1000-fold weight excess of poly dI:dC over the specific target DNA duplex. The reactions were incubated for 30 min at 25 °C, followed by the addition of a one-half volume of loading buffer (50% glycerol and 0.05% (w/v) bromphenol blue). The free DNA was separated from the enzyme-bound DNA duplex by electrophoresis through an 8% native polyacrylamide gel in 45 mM Tris borate (pH 7.5) 1 mM EDTA for 3 h at 120 V. Free DNA and enzyme-bound DNA complexes were visualized by autoradiography of the wet gels using Hyperfilm (Amersham Pharmacia Biotech) x-ray film. The binding data was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA). The data were plotted and fit to a hyperbolic curve function using Kaleidagraph (Synergy Software, Reading, PA). The K_D values were determined to be the enzyme concentration at which 50% of the substrate was shifted.

Dimer-specific Nicking Activities—Oligonucleotides (49-mers) containing either a site-specific *cis-syn, trans-syn* I, or *trans-syn* II thymine dimer were provided generously by Colin Smith and John-Stephen Taylor (Washington University, St. Louis, MO) with the sequence 5′-AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT3′ (23, 25). The underlining shows the position of the dimer. These DNAs were ^{32}P-labeled on the 5′-end with T4 polynucleotide kinase and annealed to an unlabeled complementary oligonucleotide. The double stranded thymine dimer-containing 49-mer was diluted with reaction buffer (25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM NaCl, and 100 μg/ml bovine serum albumin), and the appropriate concentrations of the T4-pdg, cv-pdg, E23D cv-pdg, or E23Q cv-pdg were added to the reactions for 30 min at 37 °C. Incision reactions were terminated by the addition of the loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromphenol blue, and 0.02% (w/v) xylene cyanol). The reactions were subsequently treated with 1 M piperidine and heated to boiling for 10 min. The purpose of the piperidine treatment was to convert any abasic sites into single strand breaks. The substrate DNAs were separated from the incision product DNAs by electrophoresis through a 15% denaturing polyacrylamide gel containing 8 M urea. The DNA bands were visualized by autoradiography of the wet gels using Hyperfilm-MP x-ray film (Amersham Pharmacia Biotech).

AP Site-specific Nicking Activity—A 49-mer oligonucleotide containing a site-specific uracil was synthesized (Midland Research) with the

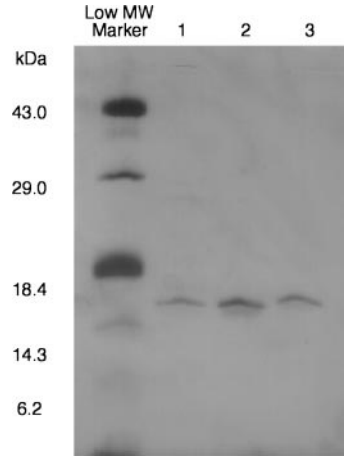


FIG. 1. **SDS-polyacrylamide gel electrophoresis analysis of cv-pdg, E23D cv-pdg, and E23Q cv-pdg after silver staining.** The *left lane* shows low range molecular mass markers (*Low MW marker*). *Lanes 1–3* show the pure fractions of E23Q cv-pdg, E23D cv-pdg, and cv-pdg, respectively.

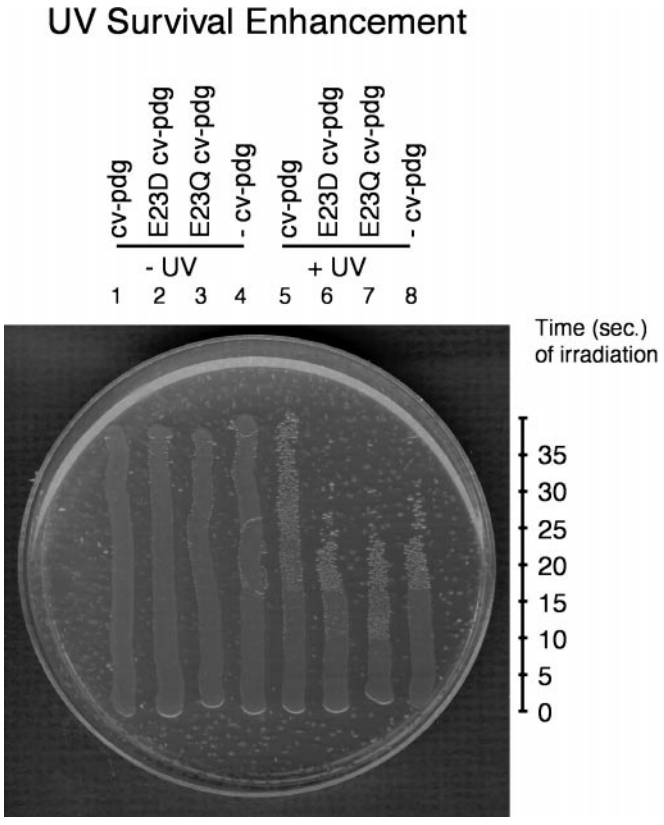


FIG. 2. ***In vivo* complementation of UV-irradiated repair-deficient *E. coli* by cv-pdg, E23D cv-pdg, and E23Q cv-pdg.** *E. coli* AB2480 (*uvrA*$^-$, recA$^-$) cells containing the pET-lla vector with cv-pdg (*lanes 1* and *5*), E23D cv-pdg (*lanes 2* and *6*), or E23Q cv-pdg (*lanes 3* and *7*) or vector alone (*lanes 4* and *8*) were grown to stationary phase and applied to an agar plate containing 100 μg/ml amp. Cells in lanes 5–8 were UV-irradiated with 254-nm light at 1 μW/cm^2 for increasing amounts of time from 0–35 s. Plates were then incubated for 12 h at 37 °C in the dark.

sequence 5′-AGCTACCATGCCTGCACGAAUTAAGCAATTCGTAATC-ATGGTCATAGCT-3′. The underlining shows the position of the uracil. This uracil-containing 49-mer was ^{32}P-labeled on the 5′-end with T4 polynucleotide kinase and annealed to its complementary oligonucleotide. The double stranded uracil-containing 49-mer was incubated with uracil DNA glycosylase (Epicentre Technologies) for 10 min at 37 °C, generating an AP site. This oligonucleotide was then diluted with reaction buffer (25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM NaCl,

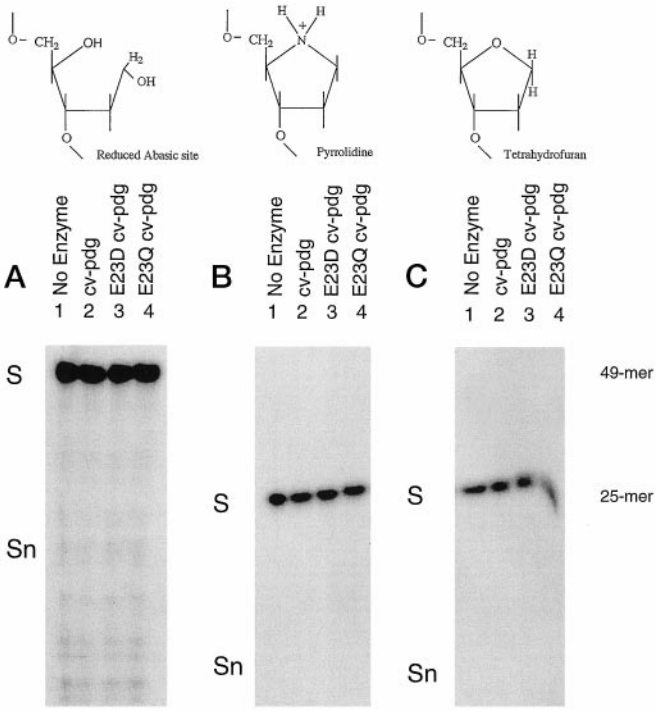


FIG. 3. **Reactions using DNAs with damaged oligonucleotides a reduced abasic site, pyrrolidine, or tetrahydrofuran.** Oligonucleotides containing centrally located damage (*A,* reduced abasic site (49-mer); *B,* pyrrolidine (25-mer); *C,* tetrahydrofuran (25-mer)) were reacted with no enzyme (*lane 1*), cv-pdg (*lane 2*), E23D cv-pdg (*lane 3*), or E23Q cv-pdg (*lane 4*) for 30 min at 37 °C. All enzymes were at 115 nM. *S* represents the substrate band, and *Sn* represents the nicked substrate (product) band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea.

and 100 μg/ml bovine serum albumin), and the appropriate concentrations of the T4-pdg, cv-pdg, E23D cv-pdg, or E23Q cv-pdg were added to the AP-containing DNA (30 min at 37 °C). The reactions were analyzed as described above.

Covalent Trapping of Imino Intermediate Using Sodium Borohydride—Oligonucleotides containing either a *cis-syn* thymine dimer or the abasic site were diluted to obtain a final substrate concentration of 0.5 nM in the reaction buffer described above. Reactions were initiated by the simultaneous addition of 100 mM $NaBH_4$ or 100 mM NaCl with 115 nM T4-pdg, cv-pdg, E23D cv-pdg, or E23Q cv-pdg. In control experiments, the substrates were preincubated with 100 mM $NaBH_4$ or 100 mM NaCl for 5 min prior to addition of the enzymes. All reactions were incubated for 30 min at 25 °C. Reactions were terminated as described above, and the trapped complexes were separated from free DNAs by electrophoresis through a 15% polyacrylamide 8 M urea denaturing gel.

RESULTS

Generation of Mutations at Glu-23 in cv-pdg and Protein Purification—Site-directed mutagenesis was carried out on the pET-11a vector containing the cv-*pdg* gene using the Quikchange mutagenesis kit (Stratagene) to introduce a codon change at Glu-23 to create either a glutamine (E23Q) or an aspartic acid (E23D). The cv-*pdg* genes were completely sequenced to verify the identity at each nucleotide. These DNAs were transformed into *E. coli* BL21DE3 for expression. The mutant proteins were expressed and purified as described under "Experimental Procedures." The purity of the protein preparations was assessed by silver staining of a 15% polyacrylamide gel. E23Q cv-pdg (Fig. 1, *lane 1*), E23D cv-pdg (*lane 2*), and wild type cv-pdg (*lane 3*) were visible without any discernable contaminants.

Relative UV Survival—The ability of cv-pdg and the E23D and E23Q mutants to enhance the survival of an *E. coli* strain that is recombination and nucleotide excision repair-deficient (*uvrA*$^-$, *recA*$^-$) was determined by challenge with increasing

doses of UV irradiation (Fig. 2). Plasmids containing the wild type and mutant cv-*pdg* genes were transformed into *E. coli* AB2480. Overnight cultures of these cells were streaked onto an agar plate and either completely shielded from UV irradiation (Fig. 2, *lanes 1–4*) or irradiated (*lanes 5–8*) for increasing times. Cells that expressed cv-pdg (*lane 5*) exhibited increased survival, whereas cells expressing the two mutants, E23D and E23Q (*lanes 6* and *7,* respectively), had survivals that were indistinguishable from cells that contained the pET11a vector alone (*lane 8*). These results demonstrate that cv-pdg led to increased survival of UV challenged bacteria, whereas the bacteria expressing cv-pdg mutants at Glu-23 resulted in no increased survival following UV challenge.

K_D Determinations—A possible explanation for the decreased UV survival of cells expressing mutant forms of cv-pdg was that these mutations altered pyrimidine dimer-specific binding. In order to estimate relative binding affinities, dissociation constants were determined for wild type and mutant enzyme on noncleavable substrate analogues. For these determinations, gel mobility shift analyses were chosen because this assay represents the standard method that is used to estimate K_D values for other glycosylase/AP lyases in the base excision repair pathway, *i.e.* endonuclease VIII (26), Fpg (27), endonuclease V (28), and MutY (29). For these assays, it was necessary to use substrates that are not catalytic substrates for these enzymes. Although previously it had been demonstrated that T4-pdg and cv-pdg could not incise DNAs containing reduced AP sites, tetrahydrofuran residues, or pyrrolidine sites (23, 28), to ensure a complete lack of activity on these substrates, cv-pdg, E23D cv-pdg, and E23Q cv-pdg were examined for residual incision activity. The data in Fig. 3 show no activity for the enzyme preparations on any of these three substrates.

Gel shift analyses were performed to determine the relative dissociation constants for the wild type and mutant enzymes on noncleaveable substrates (Table I). Labeled DNAs were incubated with increasing concentrations of enzymes for 30 min at 25 °C and analyzed by native polyacrylamide gel electrophoresis. The wild type cv-pdg showed comparable dissociation constants to those determined for the E23D cv-pdg mutant on all of its noncleavable DNA substrates. These data strongly suggest that the decreased UV survival cannot be attributed to a loss of specific DNA binding. However, the E23Q cv-pdg mutant was found to have a reduced binding of 20–100-fold on all the substrates tested. These data indicate that reduced affinity for specific substrates may significantly contribute to reduced UV survival. In comparison, the T4-pdg mutant E23Q did not exhibit significant loss in binding to these noncleavable substrates, suggesting significant differences in the details of the damage recognition site.

Pyrimidine Dimer Substrate Specificity—To characterize the dimer substrate specificity, *in vitro* incision assays were used to determine the relative nicking activity of the wild type and mutant cv-pdg enzymes on separate DNAs, each containing one of the three isomers of the cyclobutane pyrimidine dimer, *cis-syn, trans-syn* I, or *trans-syn* II. Seven concentrations of T4-pdg, cv-pdg, and E23D cv-pdg were assessed for activity on the *cis-syn* thymine dimer 49-mer (Fig. 4). To monitor every glycosylase event and not only the combined glycosylase and concomitant AP lyase activities, the reactions were treated with piperidine prior to electrophoretic separation of substrates and nicked products. The relative activity between these proteins can be compared by the enzyme concentration that converted 50% of a 0.5 nM substrate into product in 30 min. These activity comparisons were made relative to the activity of the wild type cv-pdg on the *cis-syn* 49-mer (Table II). Wild type cv-pdg had a relative activity of 0.09 nM, T4-pdg was 0.19 nM, and E23D cv-pdg was 0.60 nM. Additionally, when these experiments were carried out without the final piperidine treatment, no decrease in the amount of product DNA generated was detected, suggesting that all the glycosylase activity was followed by a concomitant AP lyase activity (data not shown). E23Q cv-pdg was found to have no detectable activity on the *cis-syn* 49-mer, even when the reaction time was increased from 30

TABLE I
Dissociation constant determination

	cv-pdg	cv-pdg E23D	cv-pdg E23Q	T4-pdg[a]	T4-pdg[a] E23D	T4-pdg[a] E23Q
				nM		
Pyr 25-mer	2.5	9.2	400	17	120	18
RAP 49-mer	10.0	9.0	200	9	40	2
THF 25-mer	12.0	5.0	280	ND[b]	180	38
CS 49-mer	ND	ND	400	ND	150	20
TSII 49-mer	ND	1.5	130	ND	ND	ND

[a] The dissociation constants for the wild type and mutant T4-pdg enzymes were determined by McCullough *et al.* (18).
[b] ND, not determined.

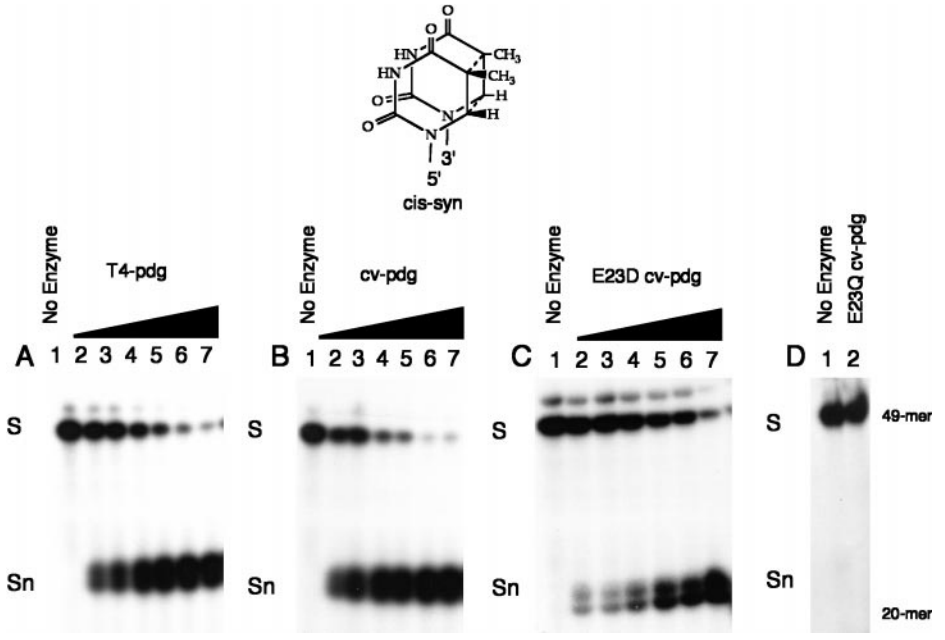


FIG. 4. ***Cis-syn* thymine dimer-specific nicking.** An oligonucleotide containing a *cis-syn* thymine dimer (0.5 nM) was reacted with increasing concentrations of T4-pdg (endonuclease V) (*A*), cv-pdg (*B*), E23D cv-pdg (*C*), and E23Q cv-pdg (*D*) for 30 min at 37 °C. *S* represents the DNA substrate band, and *Sn* represents the nicked substrate (product) band. Following the 30-min incubation, each reaction was treated with piperidine to convert any DNAs containing AP sites to the incised product band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea. *Lanes 1–7* are at 0, 0.094, 0.19, 0.37, 0.75, 1.5, and 3.0 nM for each enzyme except E23Q cv-pdg (*D*), for which *lanes 1* and *2* are 0 and 3.0 nM.

min to 6 h (data not shown). Consistent with this finding, the binding affinity of E23Q for DNA containing a *cis-syn* dimer was very weak (400 nM.) (Table I), a value approaching the affinity for undamaged DNA.

Additionally, five concentrations of T4-pdg, cv-pdg, and E23D cv-pdg were analyzed for their activity on another isomer of the thymine dimer, *trans-syn* II (Fig. 5). Relative to the activities observed on the DNA containing the *cis-syn* dimer, the activity on this substrate was reduced for T4-pdg (6.0 nM) and cv-pdg (1.5 nM) (Table I). However, the most significant differential effect was evident when comparing the activities of the E23D cv-pdg enzyme on the *trans-syn* II and *cis-syn* isomers, in which the E23D cv-pdg lost all detectable activity on the *trans-syn* II isomer (Fig. 5*D*). The E23Q cv-pdg showed no activity on the *trans-syn* II dimers, data that are consistent with its lack of activity on the *cis-syn* dimer (Fig. 4*D*). Because cv-pdg mutants E23D and E23Q did not incise DNA containing the *trans-syn* II dimer, relative binding affinities were determined using this DNA as a substrate (Table I). The E23D mutant bound with a 1.5 nM affinity (Fig. 6) and yet was unable to cleave this substrate. The E23Q mutant displayed a significantly weaker affinity at 130 nM (Table I).

T4-pdg, cv-pdg, E23D cv-pdg, and E23Q cv-pdg were also examined for activity on a third thymine dimer isomer, *trans-syn* I. Because it had been previously reported that this preparation of *trans-syn* I dimer was contaminated with 1–2% *cis-syn* dimer (23), when each of the enzymes were reacted with this substrate, approximately 5% of the DNAs were incised (Fig. 7*A*). These data were consistent with incision at the *cis-syn* contaminant and not the *trans-syn* I substrate. However, to confirm this interpretation, kinetic experiments were carried out at a very high enzyme concentration (175 nM) (Fig. 7*B*). These results demonstrated no increase in product DNAs beyond that observed in Fig. 7*A*. Taken together, these data suggest that T4-pdg, cv-pdg, and E23D cv-pdg do not have substantial activity on DNA containing the *trans-syn* I isomer.

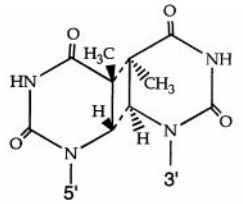
TABLE II
Specific activity of T4-pdg, cv-pdg, and cv-pdg mutants

	T4-pdg	cv-pdg	E23D cv-pdg	E23Q cv-pdg
			nM	
CS 49-mer	0.19	0.09	0.60	NA[a]
TSI 49-mer	NA	NA	NA	NA
TSII 49-mer	6.0	1.5	NA	NA
AP 49-mer	0.15	0.17	0.18	>115
RAP 49-mer	NA	NA	NA	NA
Pyr 25-mer	NA	NA	NA	NA
THF 25-mer	NA	NA	NA	NA

[a] NA, no detectable activity.

AP Site-specific Nicking Activity on Synthetic Oligonucleotides—In order to determine the ability of T4-pdg, cv-pdg, E23D cv-pdg, and E23Q cv-pdg to incise DNA at AP sites, an AP-containing oligonucleotide was prepared as described under "Experimental Procedures." This ^{32}P-labeled duplex AP-containing 49-mer (0.5 nM) was incubated with seven concentrations of the pure enzymes for 30 min at 25 °C (Fig. 8). The relative activities of T4-pdg (0.15 nM), cv-pdg (0.17 nM), and E23D cv-pdg (0.18 nM) on the AP-containing 49-mer were very consistent, whereas the E23Q cv-pdg mutant, which had no activity on DNA containing cyclobutane dimers (Fig. 4*D*), had very low activity (>115 nM) (Table II and Fig. 9*A*). This approximately 1000-fold decreased activity of the E23Q cv-pdg mutant, relative to either of the two wild type enzymes, was confirmed by kinetic experiments (Fig. 9*B*).

Covalent Trapping of the Imino Intermediate via $NaBH_4$—Previously, it was determined that other glycosylase/AP lyases that function in the base excision repair pathway are able to cleave the phosphodiester backbone via a β-elimination reaction through the formation of an imino intermediate (17, 30). In support of this conclusion was the ability of these enzymes to be covalently trapped on DNAs containing pyrimidine dimers and abasic sites when reactions were carried out in the presence of a strong reducing agent, $NaBH_4$. Thus, the ability to form and trap this imino intermediate was assessed for all the enzymes on both the *cis-syn*- and AP site-containing 49-mers (Fig. 10, *A* and *B,* respectively). The reactions shown in the first lane of each enzyme grouping (Fig. 10, *A, lanes 2, 5, 8,* and *12,* and *B, lanes 2, 5, 8,* and *11*) were conducted such that the $NaBH_4$ and the appropriate enzyme at 115 nM were simultaneously added to the substrate DNA. The trapped complexes were observed as having reduced mobility due to the enzyme-DNA covalent link-



trans-syn II

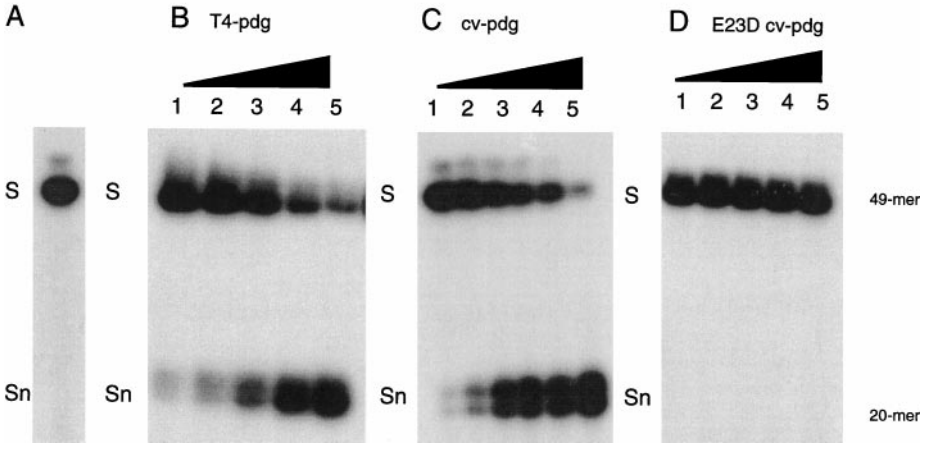


FIG. 5. ***Trans-syn* II thymine dimer-specific nicking activity.** An oligonucleotide containing a *trans-syn II* thymine dimer (0.5 nM) was reacted with increasing concentrations of T4-pdg (*B*), cv-pdg (*C*), and E23D cv-pdg (*D*) for 30 min at 37 °C. *A* shows control *trans-syn* II dimer DNA without the addition of any enzyme. *B–D* (*lanes 1–5*) show reactions at 0.37, 0.75, 3.0, 12.0, and 48.0 nM, respectively, for each enzyme. *S* represents the substrate band, and *Sn* represents the nicked substrate (product) band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea.

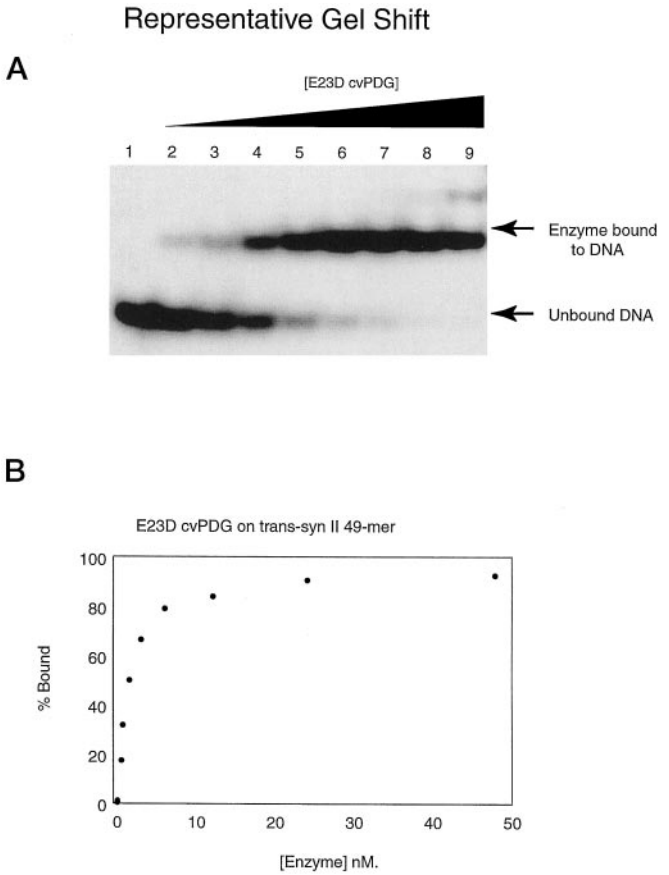


FIG. 6. **Gel Shift of E23D cv-pdg on *trans-syn II* 49-mer.** *A,* oligonucleotides containing a *trans-syn II* thymine dimer (20 pM) were incubated with increasing concentrations of E23D cv-pdg for 30 min at 25 °C. The products were separated by electrophoresis through an 8% native polyacrylamide gel. The wet gels were visualized on x-ray film, and the substrate and product bands were quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. *B,* the data were plotted using Kaleidgraph software, and the K_D was determined by fitting the data to a hyperbolic function, plotting percentage shifted *versus* enzyme concentration, and determining the enzyme concentration that resulted in a 50% substrate shift.

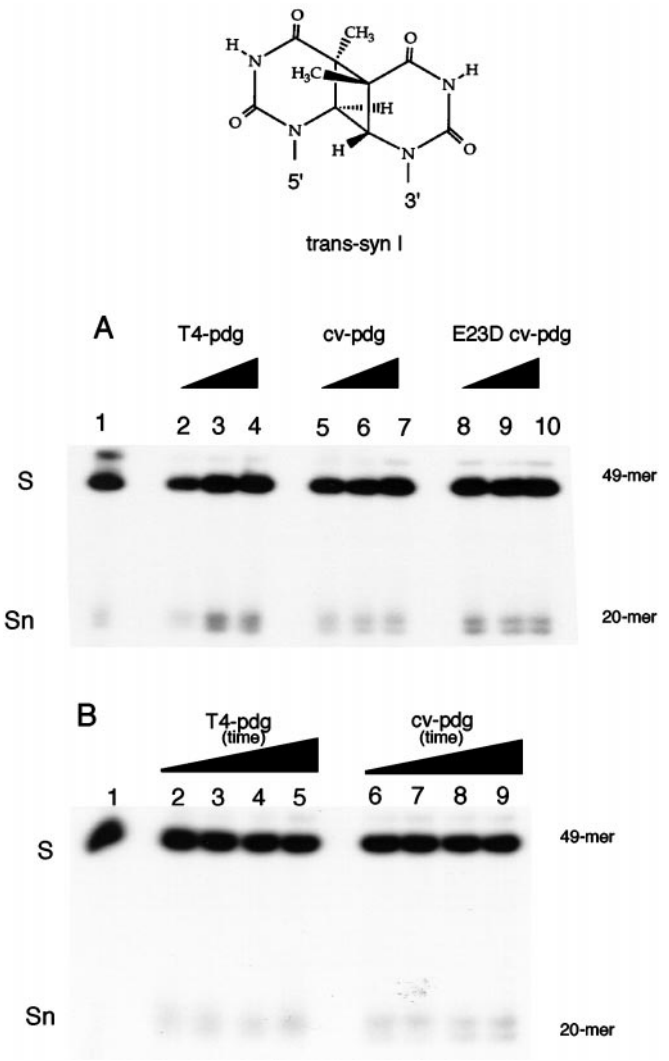


FIG. 7. ***Trans-syn I* thymine dimer-specific nicking activity.** An oligonucleotide containing a *trans-syn I* thymine dimer (0.5 nM) was reacted with increasing concentrations of T4-pdg, cv-pdg, and E23D cv-pdg for 30 min at 37 °C. *A, lane 1,* control *trans-syn* I dimer with no added enzyme; *lanes 2–4,* T4-pdg; *lanes 5–7,* cv-pdg; *lanes 8–10,* E23D cv-pdg; each at 12, 48, and 192 nM, respectively. *B* shows a kinetic analysis of incision of DNA containing *trans-syn* I dimer (0.5 nM) with T4-pdg (115 nM) (*lanes 2–5*) and cv-pdg (115 nM) (*lanes 6–9*). Time points were taken at 0, 1, 2, 4, and 6 h, for T4-pdg and cv-pdg (*lanes 2–5* and *6–9,* respectively). *S* represents the substrate DNA band, and *Sn* represents the nicked substrate (product) band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea.

age upon $NaBH_4$ reduction. Significant amounts of trapped complexes were evident for T4-pdg and cv-pdg, whereas a small amount of trapped complex was visible for the E23D cv-pdg mutant on the *cis-syn* 49-mer. No trapped complex was evident for the E23Q cv-pdg mutant. The DNAs shown in the second lane of each enzyme grouping (Fig. 10, *A, lanes 3, 6, 9,* and *13,* and *B, lanes 3, 6, 9,* and *12*) show the data from experiments in which the substrates were preincubated with 100 mM $NaBH_4$ for 5 min prior to the addition of enzyme. This preincubation step did not significantly affect the amount of pyrimidine dimer trapped complex seen as an altered mobility in the gel for the T4-pdg and cv-pdg, whereas no trapped complexes were observed for either the E23D or E23Q cv-pdg mutant.

When the abasic site containing 49-mer was used as a substrate, T4-pdg, cv-pdg, and E23D cv-pdg were all trapped, a result consistent with the incision data (Fig. 10*B*). No trapped complex appeared with the E23Q cv-pdg mutant. Also as expected, preincubation of the AP-containing DNA with $NaBH_4$ resulted in an inability to trap covalent complexes when the enzymes were added after the 5 min preincubation. During the initial 5-min incubation, all AP sites were reduced and thus not subject to covalent trapping by $NaBH_4$. The remainder of the experiments were controls to show activity on the *cis-syn* and AP site containing 49-mers.

DISCUSSION

In the initial description of cv-pdg, *Chlorella* cells that were infected with *Paramecium bursaria Chlorella* virus-1 were found to have dimer-specific nicking activity similar to the activity exhibited by T4-pdg (7, 23). The 41% amino acid identity between these two enzymes, as well as a basic similarity in substrate activity, led to the formation of a structural model for cv-pdg based on the x-ray crystal structures of T4-pdg and T4-pdg bound to a dimer-containing oligonucleotide. Through analysis of this structural model, site-directed mutants were designed to examine the catalytic mechanism of cv-pdg. Due to the proximity of Glu-23 to the dimer in the modeled structure and the mechanistic information available about other glycosylase/AP lyases, this residue was hypothesized to serve as the acidic residue necessary for glycosidic bond destabilization. This allows the C1′ of the deoxyribose sugar of the 5′ pyrimidine to become vulnerable to attack from a primary or second-

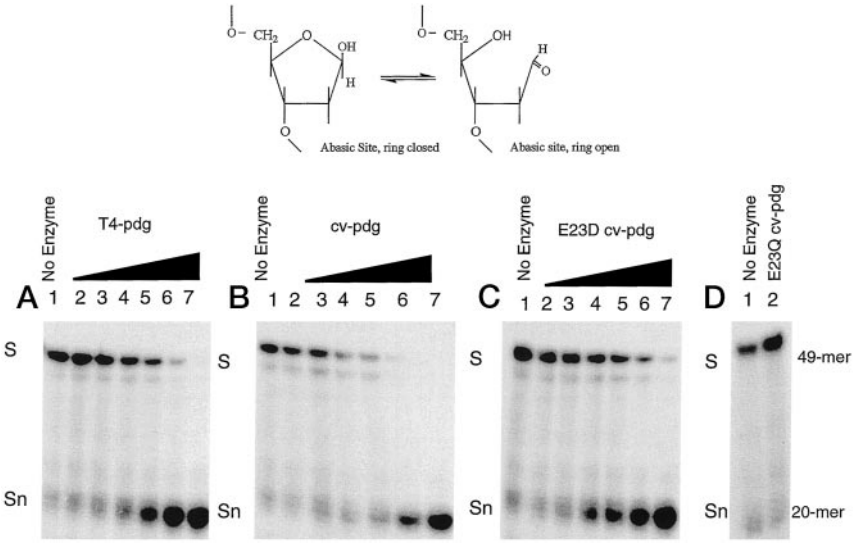


FIG. 8. **AP site-specific nicking.** An oligonucleotide containing an abasic site (0.5 nM) was reacted with increasing concentrations of T4-pdg (*A*), cv-pdg (*B*), E23D cv-pdg (*C*), and E23Q cv-pdg (*D*) for 30 min at 25 °C. The concentrations of T4-pdg and cv-pdg were 0, 0.023, 0.047, 0.094, 0.19, 0.37, and 0.75 nM for *lanes 1–7,* respectively; concentrations of E23D cv-pdg were 0, 0.047, 0.094, 0.19, 0.37, 0.75, and 1.5 nM for *lanes 1–7,* respectively; and concentrations of E23Q cv-pdg were 0 and 1.5 nM, for *lanes 1* and *2,* respectively. *S* represents the substrate DNA band, and *Sn* represents the nicked substrate (product) band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea.

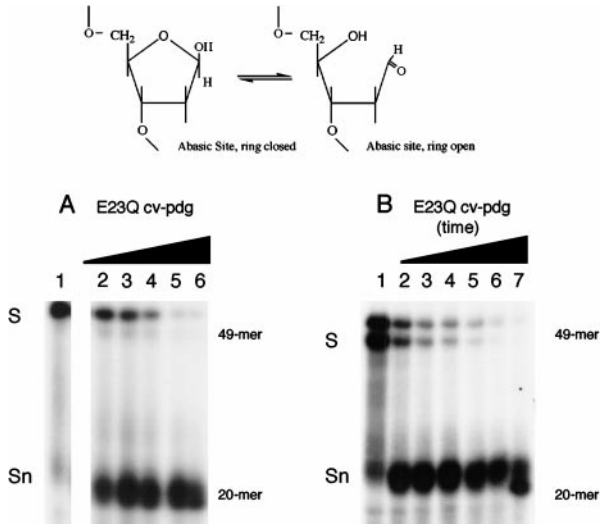


FIG. 9. **E23Q cv-pdg incision of a synthetic oligonucleotide containing an abasic site.** An oligonucleotide containing an abasic site (0.5 nM) was reacted with increasing concentrations of E23Q cv-pdg for 30 min at 25 °C. *A, lanes 1–6* show increasing concentrations of E23Q cv-pdg 0, 57.5, 115, 172.5, 230, and 287.5 nM, respectively. Due to the relatively high enzyme concentration necessary to achieve a small amount of nicked substrate, a kinetic experiment (*B*) was carried out in which 0.5 nM DNA was reacted with 115 nM E23Q cv-pdg for increasing amounts of time. *Lane 1* shows the reaction at 0 h, and *lanes 2–7* show the DNA products at 0.5, 1, 2, 3, 4, and 5 h, respectively. *S* represents the substrate DNA band, and *Sn* represents the nicked substrate (product) band. The reaction products were separated by electrophoresis on a 15% polyacrylamide denaturing gel containing 8 M urea.

ary amine. Mutations in cv-pdg were designed to assess the necessity of the acidic charge at this position (E23Q) and the importance of side chain positioning in the active site (E23D).

Initially, the wild type and mutant cv-pdg enzymes were assessed for their ability to enhance the survival of recombinational repair and nucleotide excision repair deficient *E. coli*. Wild type cv-pdg enhanced survival over that of the plasmid alone, whereas both of the mutants resulted in survival levels corresponding to that seen with the plasmid alone. The inability of either the E23D or E23Q cv-pdg mutants to enhance survival of repair-deficient *E. coli* are consistent with the results obtained when the same mutations were created in T4-pdg. However, this lack of biological activity is not completely consistent with the relative *in vitro* activities of the E23D mutants of cv-pdg *versus* T4-pdg, in which the cv-pdg mutant retained substantial *cis-syn* dimer-specific nicking activity. An understanding of this difference may lie in the differential specific activities of the two mutants. Although similar differences in *in vitro versus in vivo* activities have been previously observed for mutants of T4-pdg that affect nontarget binding, there is no indication that the E23D cv-pdg mutant has been affected in the mechanism of target site location (31–33). However, these differential survivals provided the first evidence that Glu-23 was going to be critical for the activity of this enzyme. This led us to perform a number of more sensitive *in vitro* assays to determine the relative binding and activity of the wild type and mutant proteins on various isomers of thymine dimers and abasic sites.

The E23Q mutant was inactive on all thymine dimer-containing substrates tested. This result indicates that the acidic nature at Glu-23 is critical to thymine dimer-specific nicking activity, although a confounding factor in this analysis was that dimer-specific DNA binding was greatly reduced in this mutant. However, the overall fold of E23Q cv-pdg is probably correct because it did have some very minor activity on AP site-containing DNA; however, even this activity could be attributed to the presence of various basic residues on the face of this DNA binding enzyme in close proximity to the AP site. Similar observations have been made for DNA ligase on AP-containing DNAs (8).

The E23D cv-pdg mutant exhibited very similar activity on AP-containing DNA to both the wild type cv-pdg and the T4-pdg controls. The activity seen on the *cis-syn* isomer of the thymine dimer was only slightly diminished for E23D when compared with native cv-pdg. This is in stark contrast to the results observed for the E23D T4-pdg mutant, which has less than 1% of the activity of the wild type T4-pdg (15). This could possibly be explained by a more flexible active site, initially hypothesized by McCullough *et al.* (23), based on the broader substrate specificity seen for cv-pdg relative to T4-pdg. An additional interesting feature of the E23D cv-pdg mutant was that it had no detectable activity on the *trans-syn* II isomer, whereas the wild type cv-pdg and T4-pdg had slightly diminished activity when compared with the *cis-syn* isomer. The *trans-syn* II thymine dimer has the 3′ thymine of the dimer flipped *trans*, which might alter the positioning of the 5′ thymine due to steric considerations. Taking into consideration that these enzymes perform their chemistry on the 5′ thymine of the dimer, this could help to explain the activity results obtained for the wild type and E23D cv-pdg. The relative activity between the *cis-syn* dimer and the *trans-syn* II isomer for cv-pdg shows approximately a 16-fold decrease. This decrease

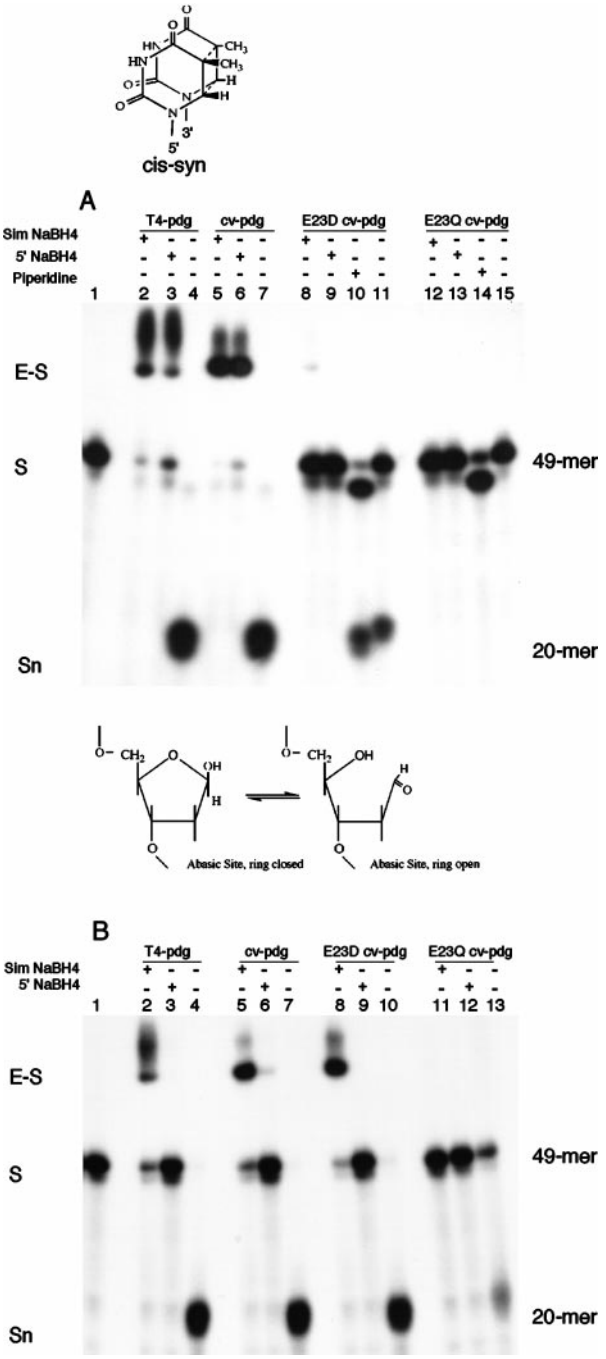


FIG. 10. **Covalent trapping on *cis-syn* dimer and AP site containing DNA.** DNAs containing either a centrally located *cis-syn* thymine dimer (0.5 nM) (*A*) or a centrally located abasic site (0.5 nM) (*B*) were incubated with 115 nM of T4-pdg, cv-pdg, E23D cv-pdg, and E23Q cv-pdg. DNAs were either preincubated with 100 mM $NaBH_4$ for 5 min prior to addition of the enzyme or added simultaneously with the enzyme. Reactions were incubated for 30 min at 25 °C. *S* represents the substrate DNA band, *Sn* represents the nicked substrate (product) band, and *E-S* represents the enzyme DNA covalent complex.

could result from a slight alteration in the active site geometry. The lack of activity of the E23D cv-pdg mutant on the DNA containing *trans-syn* II dimers may simply reflect that by shortening the side chain by one carbon, the carboxyl side chain can no longer facilitate glycosyl bond scission but is still appropriately positioned to catalyze the β-elimination reaction. In the case of the *trans-syn* I isomer, the 5′ thymine is moved into a *trans* position. The 5′ thymine being in a significantly different position could account for the lack of activity of any of these glycosylase/AP lyases, as well as their mutants.

Binding studies were performed to ensure that the mutants that had lost activity had not lost the ability to bind to the DNA. The E23D cv-pdg mutant bound to a *trans-syn* II dimer with a dissociation constant that showed tighter affinity than that observed for cv-pdg bound to a *cis-syn* thymine dimer or some of the noncleavable substrates. These data indicate that the inability to incise the DNA at *trans-syn* II dimers was not a result of a decreased binding to the substrate DNA. The E23D cv-pdg enzyme was able to bind to all noncleavable substrates with approximately equal affinity. These affinities were significantly tighter than that measured for the E23D T4-pdg binding to noncleavable substrates, thus supporting the hypothesis that the active site for cv-pdg may be more flexible.

In the reaction mechanism hypothesized for glycosylase/AP lyases in the base excision repair pathway, there is a combined action requiring an acidic residue and a primary amine. It is hypothesized that Glu-23 is the acidic residue that contributes to glycosidic bond destabilization, functioning either at the ring oxygen of the deoxyribose sugar or the C2 of the thymine. Following this destabilization, a primary amine catalyzes a nucleophilic displacement reaction at the C1′ of the deoxyribose sugar. In this reaction mechanism, the enzyme DNA complex forms an imino intermediate, which would be reduced to a covalent complex in the presence of a strong reducing agent. Relevant to these hypotheses, the E23Q cv-pdg mutant could not be trapped on either a *cis-syn* dimer-containing DNA or an abasic site-containing DNA, suggesting that the acidic character at this position is essential to facilitate the reaction mechanism. The E23D cv-pdg mutant could be trapped on the abasic site-containing DNA to levels approximately the same as those of the wild type cv-pdg and T4-pdg. However, the ability to form a trapped complex for the E23D cv-pdg mutant was significantly reduced when compared with cv-pdg. This result is somewhat surprising, but it may be due to the decrease in the activity that is seen between these two enzymes.

The site-directed mutations that were examined in this study give significant insight into the mechanism of this glycosylase/AP lyase. It is evident that the presence of a carboxylate-containing side chain at the 23rd position is essential to both the glycosylase and the AP lyase activities of this enzyme. There must be a relatively flexible active site in this enzyme as the E23D mutant retains activity on a *cis-syn* thymine dimer and an abasic site. The ability to trap the wild type cv-pdg and the E23D cv-pdg mutant onto the DNA through the formation of a covalent complex using a strong reducing agent suggests that this glycosylase AP lyase follows the mechanism hypothesized for T4-pdg (17).

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