Characterization of a Novel cis-syn and trans-syn-II Pyrimidine Dimer Glycosylase/AP Lyase from a Eukaryotic Algal Virus, Paramecium bursaria chlorella Virus-1*

(Received for publication, December 11, 1997, and in revised form, March 16, 1998)

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Endonuclease V from bacteriophage T4, is a cis-syn pyrimidine dimer-specific glycosylase. Recently, the first sequence homolog of T4 endonuclease V was identified from chlorella virus Paramecium bursaria chlorella virus-1 (PBCV-1). Here we present the biochemical characterization of the chlorella virus pyrimidine dimer glycosylase, cv-PDG. Interestingly, cv-PDG is specific not only for the cis-syn cyclobutane pyrimidine dimer, but also for the trans-syn-II isomer. This is the first trans-syn-II-specific glycosylase identified to date. Kinetic analysis demonstrates that DNAs containing both types of pyrimidine dimers are cleaved by the enzyme with similar catalytic efficiencies. Cleavage analysis and covalent trapping experiments demonstrate that the enzyme mechanism is consistent with the model proposed for glycosylase/AP lyase enzymes in which the glycosylase action is mediated via an imino intermediate between the C1’ of the sugar and an amino group in the enzyme, followed by a β-elimination reaction resulting in cleavage of the phosphodiester bond. cv-PDG exhibits processive cleavage kinetics which are diminished at salt concentrations greater than those determined for T4 endonuclease V, indicating a possibly stronger electrostatic attraction between enzyme and DNA. The identification of this new enzyme with broader pyrimidine dimer specificity raises the intriguing possibility that there may be other T4 endonuclease V-like enzymes with specificity toward other DNA photoproducts.

DNA damage caused by ultraviolet (UV) light is of great interest because it may lead to mutations, carcinogenesis, and cell death (reviewed in Refs. 1 and 2). UV-induced DNA damage occurs frequently in DNA as the bases of nucleic acids absorb light in a range coincident with that of natural sunlight, thus making them very susceptible to photochemically induced alterations (3, 4). Exposure of DNA to UV light produces several types of photoproducts including 6-4 photoproducts, photohydrates, and cyclobutane pyrimidine dimers (CPDs) (4, 5). At short wavelengths of UV light, the CPD photoproducts are the most common. Two different classes of CPDs are produced by UV light, the predominant form being the cis-syn isomer and a minor form, the trans-syn isomer being formed at a rate of about 2% that of the cis-syn isomer (Fig. 1) (6). The trans-syn isomer may exist as two stereoisomers, the trans-syn-I or the trans-syn-II (7). It has not been determined which of these trans-syn isomers are formed in DNA in vivo. The repair and mutagenicity of most of these photoproducts have been extensively investigated and reviewed (8).

A base excision repair pathway for removing pyrimidine dimers is found in the bacteriophage T4. The phage T4 genome contains the denV gene which encodes T4 endonuclease V, a well-characterized DNA base excision repair enzyme (reviewed in Ref. 9). T4 endonuclease V is a 16-kDa protein with cis-syn pyrimidine dimer-specific glycosylase activity and a concomitant AP lyase activity.

The DNA glycosylase/AP lyase activity of T4 endonuclease V exhibits salt-dependent cyclobutane pyrimidine dimer site cleavage kinetics (10–12), reminiscent of the processive kinetics of polymerases, regulatory proteins, and other DNA-reactive enzymes such as the restriction endonuclease EcoRI (13). Once associated with DNA, the enzyme is thought to “scan” the DNA molecule (via some, in general, unspecified mechanism) until binding to a target site, e.g. a pyrimidine dimer in the case of T4 endonuclease V. The kinetic consequence of such a method of substrate site binding is to reduce the dimensional-ity of the diffusive encounter between target and enzyme active site. At salt concentrations corresponding to sodium ion concentrations below 40 mM, T4 endonuclease V follows “processive” kinetics. At higher salt concentrations T4 endonuclease V follows “distributive” kinetics, where the normal three-dimensional diffusive binding to substrate is maintained (11, 14).

Biochemical studies have demonstrated that the N-terminal αNH₂ group of Thr-2 (Met-1 is removed during cellular processing) mediates the glycosylase action by forming an imino intermediate with the C1’ of the 5’ sugar of the cis-syn CPD (15–17). Phosphodiester bond scission between the pyrimidines of a CPD occurs by a β-elimination reaction resulting in a 5’-phosphate and a 3’-α,β-unsaturated aldehyde termini (18–20).

The abbreviations used are: CPD, cyclobutane pyrimidine dimer; AP, abasic site; BSA, bovine serum albumin; bp, base pair(s); MES, 2-(N-morpholino)ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBCV, Paramecium bursaria chlorella virus-1.

* This work was supported in part by National Institutes of Health Grants F32 ES05780–01 (to A. K. M.), RO1 ES04091 and F30 ES06676 (to R. S. L.), CA 40693 (to J.-S. T.), and GM 32441 (to J. V. E.), and by the Sealy and Smith Foundation, administered by the Sealy Center for Structural Biology at University of Texas Medical Branch. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Similar activities have been identified in only a few microorganisms to date. Interestingly, two different cis-syn CPD enzymes have been isolated from the bacterium Micrococcus luteus and the genes for both enzymes have recently been cloned. One encodes a 31-kDa protein with homology to the endonuclease III family of DNA repair enzymes (21) and the other, uweA, encodes an 18-kDa protein (22). Even though the uweA gene product has 27% amino acid identity with T4 endonuclease V, this represents at best, a marginal overall similarity and several key residues are not conserved. Thus, despite intense efforts to identify T4 endonuclease V homologs, it was only recently that the first true sequence homolog was found.

The first homolog of T4 endonuclease V was identified from the virus, PBCV-1 (Paramecium bursaria chlorella virus-1) (23) and has been shown to initiate pyrimidine dimer repair (24). PBCV-1 is the prototype virus of the family Phycodnaviridae; these polyhedral viruses contain large (>300 kilobase), linear, nonpermuted double-stranded DNA genomes with cross-linked hairpin ends (reviewed in Ref. 25 and 26). These viruses, which have been isolated from freshwater around the world, infect certain strains of unicellular, eukaryotic, chlorella-like green algae which normally exist as endosymbionts in the protozoan Paramecium bursaria. Interestingly, these viruses encode numerous DNA methyltransferases and restriction endonucleases as well as the T4 endonuclease V homolog, a host-independent DNA UV repair enzyme. This is the first example of a virus infecting a eukaryotic cell that encodes a host-independent UV DNA repair enzyme. This is significant because most viruses rely on host enzymes to repair their DNA, a process termed host cell reactivation (8).

The pyrimidine dimer-specific cleavage activity was present in extracts from PBCV-1 infected Chlorella NC64A cells, but not in extracts from uninfected Chlorella or isolated virions (24). The pyrimidine dimer-specific activity is expressed shortly after virus infection of the host Chlorella (24). The gene (cv-pdg) encoding the T4 endonuclease V homolog was cloned, and it enhanced UV radiation resistance in repair-deficient Escherichia coli cells.

The predicted amino acid sequence of the cv-pdg protein is 41% identical to T4 endonuclease V, and potential key active site amino acid residues are conserved, as well as domains suspected to be critical for the recognition of cyclobutane pyrimidine dimers. Homology modeling studies using the coordinates of the T4 endonuclease V x-ray crystallographic structure (27, 28) suggest a high degree of structural homology. Here we present the biochemical characterization of cv-pdg, the first pyrimidine dimer specific-glycosylase from a virus infecting a eukaryotic cell.

**Experimental Procedures**

Polymerase Chain Reaction Cloning and Overexpression of the PBCV-1 pdg Gene—The entire pdg gene (open reading frame A50L) of 426 bp was amplified from PBCV-1 genomic DNA using the polymerase chain reaction. The polymerase chain reaction primers were 5'-AGCTCATGGTGTAATCATGGTC-9 and 5'-GGACACGTGTGAATCTCGTACCGG and 5'-ACTGCGTGTAATCATGGTC-9. Specific polymerase chain reaction conditions can be obtained from the authors on request. The amplified DNA was cloned using a pCRII vector (Invitrogen) and confirmed by DNA sequencing analysis (Perkin Elmer/Applied Biosystems). The primers used in amplifying the target DNA introduced a unique NdeI site at the initiator codon and a unique HindIII site beyond the termination codon (TAA). These sites were utilized to create a T7-based bacterial expression plasmid in pET-11a (Novagen). After induction with isopropyl-1-thio-β-D-galactopyranoside, protein expression was analyzed by polyacrylamide gel electrophoresis.

**Purification of the cv-pdg Protein**—E. coli BL21 (DE3) cells (34 g) containing the overexpressed PBCV-1 pdg gene product were resuspended in buffer containing 25 mM sodium phosphate (pH 6.8) and 1 mM EDTA (Buffer A), and disrupted in a French Press. Cell extracts were centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatant was loaded onto a 400-ml single-stranded DNA-agarose column, prepared as described previously (15), and equilibrated in 25 mM sodium phosphate (pH 6.8), 50 mM NaCl, and 1 mM EDTA. Following a 1500-m1 wash (25 mM sodium phosphate (pH 6.8), 0.2 M NaCl, and 1 mM EDTA), 8-ml fractions were collected over a 1-liter gradient from 0.2 to 2.0 M NaCl. Fractions from the single-stranded DNA-agarose column were assayed for pyrimidine dimer-specific DNA nicking activity using a 5'-end labeled 49-base pair cis-syn thymine dimer-containing DNA (CS-49) as described previously (29). Each fraction was incubated with 0.25 mM dimer-containing duplex DNA in 25 mM sodium phosphate (pH 6.8), 100 mM KC1, 1 mM EDTA, and 100 μg/ml bovine serum albumin (BSA) for 30 min at 37 °C. The appearance of a 20-bp product was detected on a 15% PAGE-urea gel.

**T4 Endonuclease V**—The T4 endonuclease V used in this study was purified from E. coli AB2480 (recA, uvrA) cells transformed with a denV expression vector as described previously (30).

**Oligonucleotides**—The 94-base oligonucleotides and the 12-base oligonucleotide containing site-specific photoproducts were prepared from characterized building blocks and consist of the following sequence: 5'-AGCTCATGGTGTAATCATGGTC-3' where x = 5'-6-dihydrouracil, was modified by Dr. Paul Doetsch (Emory University, Atlanta, GA). A 49-mer was purchased from Midland Certified Reagent Co. (Midland, TX) with the same sequence as the 49-mer photoproducts except with a uracil at position 21 and a thymine at position 22. The U49-mer was treated with 0.5-1.0 units of uracil DNA glycosylase (Epigenent Technologies, Madison, WI) for 20 min at 37 °C to generate a centrally located abasic site (AP). Complementary sequences were synthesized in the Molecular Biology Core Facility at the UTMB NIEHS Center, using standard procedures, and the deprotected oligonucleotides were electrophoretically purified on denaturing polyacrylamide gels. All oligonucleotides containing the photoproducts, other modifications, or control sequence were γ-32P-labeled (λ-32P)ATP, 3000 Ci/mmol, NEN Life Science Products) on the 5'-end with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) following standard procedures and annealed to their complementary strands to form duplex DNA. The DNA concentrations were determined by absorption at 260 nm (31). Plasmid DNA designated ALIA-5317 (4460 bp), was obtained from the UTMB NIEHS Center, Molecular Biology Core Laboratory, and was purified by CsCl gradient centrifugation.

**Enzyme Activity Assays**—Single-stranded or duplex DNAs (0.5-1 nM) containing the modifications were incubated with the indicated amounts of T4 or T4 endonuclease V in the standard reaction buffer containing 25 mM sodium phosphate (pH 6.8), 100 mM NaCl, 1 mM EDTA, and 100 μg/ml BSA for 30 min at 37 °C. An equal volume of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue) was added to the reactions and the samples heated (except for AP-containing DNA) to 90 °C for 2 min prior to loading onto a 15% denaturing polyacrylamide gel (8 x urea). The gel was electrophoresed in TBE buffer (90 mM Tris borate, 2 mM EDTA, pH 8.0). Where indicated, 1 μl piperidine was added to the reaction and incubated for 30 min at 90 °C prior to the addition of gel loading buffer.

2 The trans-syn thymine dimer-containing 49-mer reported in the previous study was erroneously assigned a trans-syn-I stereochemistry when in fact it has a trans-syn-II stereochemistry. A manuscript detailing the synthesis of trans-syn-I and trans-syn-II building blocks and x-ray crystallographic characterization of intermediates in their synthesis is in preparation (J. S. Taylor, manuscript in preparation).
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Bands were visualized by autoradiography of wet gels using Hyperfilm-MP x-ray film (Amersham). Product bands were quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA).

**pH Optimum and Salt Effects** — pH optimization and salt experiments were performed on the cis-syn-49 bp DNA. The DNA (0.5 µM) was incubated with 50 µM enzyme in the indicated buffer (total volume 20 µl) at 1 h at room temperature. Aliquots (10 µl) were incubated with 1 M piperidine for 30 min at 37 °C and then added to formamide-containing dye (95% [v/v] formamide, 20 mM EDTA, 0.02% [w/v] xylene cyanol, and 0.02% [w/v] bromphenol blue). The remaining 10-µl aliquots were added to formamide-containing dye and placed in a dry ice-ethanol bath. Samples were heated for 2 min at 90 °C and loaded onto a 15% denaturing polyacrylamide-urea gel (8 M urea) and electrophoresed in TBE buffer for 3 h at 20 watts. Product bands were quantitated as described above. The buffers used were sodium acetate (pH 4.5 and 5.0), MES (pH 5.0 and 6.0), Hepes (pH 6.0–8.0), sodium phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0 and 8.5), CHES (pH 8.5 and 9.0), and CAPS (pH 10.0).

Covalent Trapping Using Sodium Borohydride — Either cis-syn or trans-syn-II duplex DNA (1 µM) was incubated with 20 nM enzyme in the standard reaction buffer in the presence of 100 mM NaBH₄ (Sigma). The reactions were incubated at 37 °C for 1 h. Reactions were added to an equal volume of gel loading buffer containing 160 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 5% (w/v) SDS, and 2% (w/v) bromphenol blue. Samples were boiled for 5 min and analyzed on a 15% SDS-PAGE gel with a 3% stacking gel. Gels were electrophoresed in a Tris-glycine-SDS buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% [w/v] SDS, pH 8.3) for 4 h at 175 volts. The bands were visualized by autoradiography as described above.

**Determination of the Number of Active Molecules of cv-PDG** — Two methods were used to determine the number of active molecules in the enzyme preparation. Active site titration experiments were performed using quantitative sodium borohydride trapping of the covalent intermediate formed during catalysis. The quantitative sodium borohydride trapping experiments were performed as described above, except the DNA was at two different concentrations, 0.5 and 1 µM. 0.25 nM enzyme was incubated with 1 nM DNA. These enzyme concentrations were determined by electrophoresis through a 15% SDS-polyacrylamide gel and silver stained.

**RESULTS**

**Overexpression and Purification of cv-PDG in E. coli** — The PBCV-1 pdg gene (open reading frame A56L) was cloned into the E. coli expression plasmid pET11a. The recombinant protein comprised about 30% of the total cellular protein after induction of the pdg gene with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and the majority of the protein was recovered in the supernatant fraction after cell disruption (Fig. 2A). This supernatant fraction was applied to a single-stranded DNA agarose column, aliquots (15 µl) of the indicated fractions were separated by electrophoresis through a 15% SDS-polyacrylamide gel and silver stained.

10 min. An equal volume of gel loading buffer was added, the samples heated to 90 °C and analyzed on a 15% polyacrylamide-urea gel. The product bands were quantitated as described above. The data were plotted as rates versus substrate concentration to ensure a hyperbolic function. Subsequently, the data were analyzed as standard Eadie-Hofstee plots (or Lineweaver-Burk plots which gave similar values) and the Kₘ and Vₘₐₓ determined accordingly.

**Rate of cv-PDG Nicking of UV-irradiated Plasmid DNA** — Plasmid DNA was irradiated and prepared as described above with the time of irradiation corresponding to the amount of dimers introduced. Reactions were performed with various NaCl concentrations in the 25 mM sodium phosphate, 1 mM EDTA buffer. The reaction mixture was prewarmed for 3–5 min at 37 °C, and the reactions initiated by the addition of 0.5 µM cv-PDG. At various times, aliquots containing 1 µg of DNA were removed and quenched by adding them to electrophoresis loading buffer and quantitated as described above.

The number of active cv-PDG molecules was also determined by burst kinetic measurements. A 4.46-kilobase plasmid was diluted in 25 mM sodium phosphate (pH 6.8) and 1 mM EDTA to a concentration of 0.242 mg/ml. The DNA solution was irradiated by short wave UV light (Damar G15T8 germicidal lamp) at 100 microwatts/cm² for 400 s to generate approximately 25 pyrimidine dimers per molecule (32, 33).

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**BSA was then added to the irradiated plasmid DNA to a final concentration of 100 µM/mL. The irradiated DNA (10 µg) was prewarmed at 37 °C in reaction buffer containing 25 mM sodium phosphate (pH 6.8). NaCl was added to produce a final Na⁺ concentration of 55 mM. Reactions were initiated with the addition of cv-PDG enzyme at the following concentrations, 0.85, 1.7, 3.4, and 6.8 nm. At the indicated times, 20 µl of the reaction mixture was removed and the reaction terminated by placing on a dry ice-ethanol bath. The gels were stained overnight in Tris-borate buffer containing 1 µM/ml ethidium bromide. The ethidium bromide-stained bands were visualized on a UV light box and photographed using the Appligene Imager Camera system. The relative amounts of each of the three forms of DNA were quantitated using the BioImage Viscage Ethroelectrophoresis Gel Analysis System Viscage version 4.6L (1992) and Whole Band Analysis version 2.4 (1991) (Millipore Corp., Ann Arbor, MI).

**Steady State Kinetic Analysis** — The cis-syn and trans-syn-II 49 bp DNAs at 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 4, and 8 nM were incubated in standard reaction buffer with 0.9 µM cv-PDG enzyme for the cis-syn DNA and in the standard reaction buffer for the trans-syn-II DNA. The 12-kDa cleavage product was incubated at 0.05, 0.1, 0.5, 1, and 2 mM and was incubated at 15 °C with 4 µl enzyme as described previously (34). The reactions containing DNA and buffer were prewarmed at 37 °C for 10 min and a zero time point was taken. Aliquots were removed at 1, 2, and 3 min for the 49 bp DNAs or at 3, 6, 9, and 12 min for the 12-bp DNA and SDS was added to 0.5% to stop the reaction. Each reaction was treated with 1 M piperidine for 30 min at 90 °C, then placed on a dry ice-ethanol bath for 10 min. An equal volume of gel loading buffer was added, the samples heated to 90 °C and analyzed on a 15% polyacrylamide-urea gel. The product bands were quantitated as described above. The data were plotted as rates versus substrate concentration to ensure a hyperbolic function. Subsequently, the data were analyzed as standard Eadie-Hofstee plots (or Lineweaver-Burk plots which gave similar values) and the Kₘ and Vₘₐₓ determined accordingly.

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**Specificity of cv-PDG** — To assess the substrate specificity of cv-PDG, oligonucleotides containing the major DNA photoproducts were synthesized, radiolabeled on the 5′-end, and annealed to the complementary DNA strand. These DNA duplexes were then incubated with cv-PDG and the formation of a 20-mer product was monitored by autoradiography and PhosphorImager analysis. As shown in Fig. 3A, cv-PDG incises the duplex DNA at the sites of cis-syn T-T (lane 7) and trans-syn-II T-T (lane 13) approximately equally; however, only a small amount of activity (35-fold less) was detected on trans-syn-I T-T (lane 10). The trans-syn-II activities were greater than T4 endonuclease V in these assays (lanes 9 and 12), with the cv-PDG nicking at trans-syn-I and trans-syn-II dimers 30–70 times more efficiently than T4 endonuclease V. These results were surprising as the T4 endonuclease V enzyme exhibits only limited catalysis (−1%) at trans-syn-II pyrimidine dimers as compared with cis-syn (35). The cv-PDG cleavage product at
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Substrate specificity of cv-PDG. Panel A, 5'-end labeled duplex DNAs (0.5–1 nM) were incubated with 200 pM cv-PDG (lanes 4, 7, 10, and 13), 200 nM endonuclease V (lanes 3 and 6), 2 nM endonuclease V (lanes 9 and 12), or no enzyme (lanes 2, 5, 8, and 11) in 25 mM sodium phosphate (pH 6.8), 100 mM NaCl, 1 mM EDTA, and 100 μg/ml BSA. Following a 30-min incubation at 37 °C, samples were analyzed on a 15% polyacrylamide-urea gel. Oligonucleotide markers are shown ranging from 10 to 32 bases (lane 1). The position of the 20-base product is indicated by the arrow. Panel B, cv-PDG activity on DNA containing abasic sites. 5'-End labeled DNA containing an abasic site, either duplex (lanes 4 and 7) or single-stranded (lanes 1–3, 5, and 6) was incubated with 10 nM cv-PDG (lanes 2 and 4), 100 nM cv-PDG (lanes 3), 100 nM endonuclease V (lanes 5 and 7), 100 nM endonuclease V (lane 6), or no enzyme (lane 1) in 25 mM sodium phosphate (pH 6.8), 100 mM NaCl, 1 mM EDTA, 100 μg/ml BSA. Following a 30-min incubation at 37 °C, the samples were analyzed on a 15% polyacrylamide-urea gel. The position of the β and δ-elimination products are indicated. Approximately 5–10% of the activity on AP sites in double-stranded DNA was broad, between pH 6.0 and 8.0 (data not shown). Interestingly, the range was somewhat narrower (pH 6.0–7.0) with the sodium phosphate buffers commonly used in these experiments than with other buffers tested including Hepes, Tris, and CHES. Both the cv-PDG glycosylase and the AP lyase activities had similar pH profiles in contrast to T4 endonuclease V, where the pH optimum for the two enzymatic activities differs, with the AP lyase functioning over a more narrow range at pH 6.5 and becoming inactive by pH 8.0 (37). Interestingly, when the effects of salt concentration were investigated, there is a sharp peak of optimal activity at 100–120 mM salt (data not shown). Thus, there appears to be a stimulation of the cv-PDG activity from low salt conditions and a dramatic inhibition at very high salt for both the glycosylase and the glycosylase/AP lyase activities. KCl appears to be slightly more stimulatory than NaCl for this enzyme.

Concerning the thermal stability of the enzyme, it is difficult to completely heat inactivate, in that it retains 10% activity even after a 20-min 90 °C heat treatment (data not shown). However, the majority of activity is lost within minutes even at 42 °C. The enzyme is stable when stored in 50% glycerol at −80 °C or −20 °C for several freeze-thaw cycles, and can be kept in buffer without glycerol at 4 °C for up to a year without loss of activity.

Evidence for an Imino Intermediate—The product observed after cleavage of the pyrimidine dimers by cv-PDG is consistent with the T4 endonuclease V reaction mechanism. T4 endonuclease V catalyzes the breakage of the N1−C1-glycosyl bond linking the 5'-thymine base to the sugar phosphate backbone, followed by a concomitant AP lyase activity which catalyzes a β-elimination reaction leaving a 3'-α,β-unsaturated aldehyde and a 5'-phosphate. Thus, we expected the catalytic mechanism for cv-PDG to proceed via an imino intermediate similar to other DNA glycosylase/AP lyases (38). To establish that cv-PDG was using this mechanism, a covalent enzyme-DNA complex was trapped by adding sodium borohydride, a reducing agent, to the reaction (17). A covalent complex was detected by denaturing polyacrylamide gel electrophoresis when cv-PDG was incubated with both pyrimidine dimer-containing duplexes (cis-syn and trans-syn-II) in the presence of sodium borohydride (Fig. 4). This covalent complex was dependent on NaBH₄, as no complex was detected on either of the dimer-containing DNAs in the presence of 100 mM NaCl (Fig. 4, lanes 2 and 6). The covalent complex formed by cv-PDG was similar...
to that created by T4 endonuclease V on the cis-syn DNA (compare lanes 3 and 4). However, like the incision activity, cv-PDG traps very efficiently on the trans-syn-II DNA, while T4 endonuclease V does not (compare lanes 7 and 8). Thus, cv-PDG cleavage at pyrimidine dimers clearly involves the formation of a Schiff base intermediate. Structural modeling studies indicate that like T4 endonuclease V, the key residues involved in this catalysis will probably be Glu-23 and the N-terminal α-amino group.

**Determination of Active Molecules**—The fraction of active molecules of cv-PDG was determined by two methods. An active site titration analysis on dimer-containing DNA was conducted using quantitative sodium borohydride trapping as described above. DNA containing a cis-syn dimer was incubated for 1 h (previously determined to allow for complete reaction), with varying amounts of enzyme in the presence of sodium borohydride. The reactions were analyzed for the number of enzyme molecules covalently trapped on the DNA. The percent of the enzyme that was trapped in the presence of excess substrate DNA is a direct measure of the number of active molecules per enzyme preparation. Under these conditions, 15% of the cv-PDG molecules were active.

In addition, a burst amplitude analysis for the enzyme preparation was performed by measuring the rate of cleavage of form I plasmid DNA. The experiment was performed at low salt concentrations to maximize the differences between the burst and steady state phases of the kinetics. Enzyme to DNA molar ratios ranging from 0.05 to 0.4 were examined, and the data were fit to the sum of two exponentials (17). The burst amplitude was taken to be the relative amplitude of the fastest exponential term. A plot of burst amplitude versus enzyme concentration indicated that 12% of the enzyme was active. Thus, based on two independent determinations, the number of active molecules in this preparation of cv-PDG was approximately 13.5%, and thus all reported concentrations for quantitative analysis reflect this activity.

**Catalytic Efficiency of cv-PDG on T-T dimer Substrates**—In order to assess the enzyme's activity on the two differentphotoproducts, a kinetic comparison was performed for the cv-PDG on the cis-syn and trans-syn-II pyrimidine dimer DNA. Standard reactions were performed, and the products were quantified by PhosphorImager analysis. Reaction rates were determined at various substrate concentrations, and the data analyzed by linear regression of Eadie-Hofstee (Fig. 5) or Lineweaver-Burk plots (data not shown). The kinetic values for cv-PDG and T4 endonuclease V (34) are shown in Table I. Interestingly, cv-PDG demonstrates similar kinetic parameters on both cis-syn and trans-syn-II dimers with the catalytic efficiency of the enzyme only varying 10-fold on the two substrates. In comparison with T4 endonuclease V on a 12-bp duplex containing a cis-syn pyrimidine dimer, the enzymes vary by 20-fold in their relative efficiency of catalysis with the cv-PDG being more efficient on this substrate. Thus, despite the structural differences, the cv-PDG enzyme cleaves at cis-syn and trans-syn-II pyrimidine dimers with similar catalytic efficiency. As observed for T4 endonuclease V, cv-PDG also has a slow turnover number. Interestingly, although the 12-bp DNA is incised, the $K_m$ for this substrate was much higher than the larger substrates (20–40 times), therefore binding studies may be more appropriately performed on longer DNAs.

**Effect of Sodium Chloride Concentration on Reaction Kinetics on Plasmid DNA**—Kinetic analyses of the cv-PDG incision of plasmids containing 10 or 25 pyrimidine dimers per molecule were done in 37, 70, 103, and 137 mM total NaCl. The rate of disappearance of form I DNA is directly proportional to salt concentration, with the highest rate of disappearance occurring at the highest concentration (see Fig. 6A). The accumulation of form III DNA (approximately linear with time) results from two dimer incision events in close proximity on the complementary strands of a single DNA molecule (10). The production of form III DNA is dependent on two catalytic events, and must lag behind the formation of form II DNA, which is produced by a single catalytic event. Form III DNA is formed at a substantial rate under processive conditions. In contrast, under distributive reaction conditions, the enzyme macroscopically dissociates from the plasmid before sampling a large fraction of the DNA length. In this case, the incision at multiple pyrimidine dimers sites on the same molecule, but on complementary strands, depends on multiple enzyme-DNA encounters. In turn, this results in slower form III DNA formation relative to form I DNA disappearance. As an interesting aside, the formation of form III molecules implies either: 1) enzyme-DNA dissociation, at least on a microscopic volume scale compared with the volume of the whole DNA molecule; 2) a dimeric enzyme protein; or 3) at least two active sites per enzyme (against
which there is substantial evidence in the case of T4 endonuclease V).

As shown in Fig. 6A, salt concentration markedly affected form III DNA formation relative to the amount of form I DNA remaining in a reaction. The amount of form III DNA produced at lower salt concentrations was much greater than at higher concentrations, relative to the remaining form I DNA. For example, at 137 mM Na$^{+}$, approximately 97% of the form I DNA had disappeared with the accumulation of only 2% form III DNA after 5 min (Fig. 6A). The degree of processivity at various salt concentrations was assessed by examining the appearance of form III DNA as a function of the rate of disappearance of form I DNA (Fig. 6B). This type of plot essentially normalizes the form III formation rate to the steady state DNA nicking rate for each salt concentration. At lower salt concentrations, the rate of form III DNA formation relative to the overall reaction rate is greater than at higher salt concentrations (Fig. 6B).

**DISCUSSION**

This report describes the characterization of a novel pyrimidine dimer-specific glycosylase/AP lyase (cv-PDG) encoded by an eukaryotic algal virus. Using oligonucleotides containing thymidylyl-(3'→5')-thymidine photoproducts, we demonstrate that cv-PDG has a high degree of specificity toward cis-syn and trans-syn-II pyrimidine dimers and abasic sites within DNA. cv-PDG activity does not require divalent cations or any cofactors, has a broad pH range, and is relatively stable and soluble under most conditions. Surprisingly, despite its overall 41% amino acid identity with and apparent structural homology with T4 endonuclease V, cv-PDG has a broader substrate specificity. In addition to glycosylase activity on cis-syn CPDs, cv-PDG cleaves the 5'-glycosyl bond at trans-syn-II pyrimidine dimers with approximately the same catalytic efficiency as the cis-syn CPD. This finding suggests that the cv-PDG active site is more flexible than endonuclease V in order to accommodate the trans-syn-II isomer (see Fig. 1).

The trans-syn-II isomer differs from the cis-syn isomer in that the 3'-thymine is locked in the opposite orientation about the glycosyl bond (syn), whereas in the trans-syn-I isomer it is the 5'-thymine that is locked in the opposite orientation (Fig. 1). These structural differences result in substantially different conformations of the dinucleotide (39–41). The fact that the trans-syn-II isomer is a substrate, and the trans-syn-I isomer is not, is consistent with the mechanism by which the T4 endonuclease V enzyme is thought to initiate repair at cis-syn dimers (9). In the first step, the enzyme displaces the 5'-thymine of the dimer by activating the thymine as a leaving group, presumably by hydrogen bonding to the O-2 carbonyl group. Since the 5'-thymine of both the cis-syn and trans-syn-II dimers have the same orientation, their O-2 carbonyl groups are in the same relative position and reside in what is formally the minor groove of the duplex. In the trans-syn-I dimer, however, the 5'-thymine is in the opposite orientation, and the O-2 carbonyl group formally resides in the major groove, and would not be accessible to hydrogen bonding by the enzyme.

The biological significance of this broader substrate specificity is not known. However, it is known that trans-syn isomers are formed in greater amounts in single-stranded DNA (6). Thus, despite the fact that cv-PDG does not cleave trans-syn dimers in single-stranded DNA in vitro, these photoproducts may be formed in transiently single-stranded regions of the viral genome such as during DNA replication or transcription and then removed from the resulting duplex regions of DNA. The frequency of trans-syn pyrimidine dimers in the viral genome is not known. Studies with a T-T dinucleotide show that the trans-syn-II isomer is a relatively minor form as compared

**FIG. 6.** Processive nicking activity of the cv-PDG on UV-induced pyrimidine dimer containing form I DNA at various NaCl concentrations. Panel A, cv-PDG (0.5 nM) was added to 1.0 μg of plasmid DNA containing 25 dimers per molecule per reaction. Each reaction contained 25 mM sodium phosphate (pH 6.8), 1.0 mM EDTA, 1.0 μg/ml BSA, and additional NaCl with the final Na$^{+}$ concentrations as indicated. Solutions were incubated at 37 °C and terminated with the addition of SDS loading buffer at the indicated times. Panel B, accumulation of form III DNA relative to disappearance of form I DNA. The fraction of linear DNA molecules (form III) is plotted versus the relative disappearance of covalently closed circular (form I) DNA for each salt concentration. The data shown is for 25 dimers per plasmid DNA molecule only: 37 mM Na$^{+}$ (circle), 70 mM Na$^{+}$ (square), 103 mM Na$^{+}$ (diamond), 137 mM Na$^{+}$ (triangle).
with the \textit{trans-syn-I} (41). However, in T-C dimers the occurrence of \textit{trans-syn-II} isomers is increased to 50\% of the \textit{trans-syn-I} (7). Neither \textit{cis-syn} nor \textit{trans-syn-I} or \textit{II} pyrimidine dimers are very mutagenic in the systems studied to date (42).\textsuperscript{3} However, most organisms have at least one, if not several, mechanisms for removing both of these types of pyrimidine dimers, including other DNA repair systems such as photolyase (43) and nucleotide excision repair (44). It may be that the effects of these photoproducts are more severe in viruses which have a specific glycosylase for their removal.

The mechanisms by which DNA repair enzymes locate the damage site within large DNA molecules can be examined by characterizing the processive nature of DNA strand cleavage as has been demonstrated for T4 endonuclease V (10–12). At higher salt concentrations, the time required for macroscopic enzyme turnover on plasmid DNA substrates was much shorter than at lower concentrations. This was interpreted as a more distributive binding to pyrimidine dimer sites on the plasmid molecules. Despite the similarity between cv-PDG and T4 endonuclease V with regard to their processive kinetics, the two enzymes differ quantitatively in their salt sensitivity. Above 40 mM sodium ion, T4 endonuclease V displays predominantly distributive kinetics (11, 12). cv-PDG, however, remains processive up to 100 mM Na\textsuperscript{+}. These results suggest that cv-PDG has a higher nonspecific binding affinity for nontarget DNA than T4 endonuclease V. The biological significance of the processive DNA nicking activity in the repair enzymes has only been demonstrated for T4 endonuclease V. T4 endonuclease V mutants, catalytically similar to wild type enzyme but with reduced processive DNA nicking activity in the repair enzymes has only been demonstrated for T4 endonuclease V. T4 endonuclease V mutants, catalytically similar to wild type enzyme but with reduced processive kinetics, have been characterized (reviewed in Ref. 45). DNA repair-deficient \textit{E. coli} expressing these T4 endonuclease V mutants showed decreased ability to repair UV-damaged genomic DNA as measured by UV survival curves; thus, there was a direct correlation between the ability to survive UV irradiation and the ability to act processively in kinetic studies (45). cv-PDG is more processive than T4 endonuclease V because higher salt concentrations were required to shift cv-PDG to the distributive mode. Consequently, cv-PDG may be more efficient in initiating base excision repair than T4 endonuclease V.

In addition to being the first \textit{trans-syn-II} specific glycosylase/AP lyase, the cv-PDG enzyme can cleave AP sites in single-stranded DNA. These novel activities of this pyrimidine dimer glycosylase may prove useful in examining the effects of these DNA modifications within the cell such as mutagenesis and strand-specific repair. The specificity for two different photo-products, \textit{cis-syn} and \textit{trans-syn} T-T dimers, will also allow for structural studies into the enzyme’s active site as compared with the well characterized T4 endonuclease V.

Finally, the identification and biochemical characterization of this new PDG enzyme suggests that T4 endonuclease V-like activities are not as unique as previously thought, and that this type of glycosylase activity, directed toward pyrimidine dimers in DNA, may be more widespread and perhaps contribute to virus survival in nature. In support of this hypothesis, Southern blot analysis of many independently isolated chlorella viruses indicate that T4 endonuclease V homologs (cv-PDG) are common in this group of viruses (24).

Acknowledgments—We thank R. Cardinal for purifying T4 endonuclease V, J. Stewart for help with the purification of cv-PDG, C. D. Kodira (NIEHS Molecular Biology Core Facility) for synthesizing oligonucleotides, M. Wang and Y. Ren for synthesizing the \textit{trans-syn-I} DNA, and members of the Lloyd laboratory for many helpful discussions.

\textsuperscript{3} J.-S. Taylor, unpublished data.