Expression, Purification, and Characterization of Ultraviolet DNA Endonuclease from *Schizosaccharomyces pombe*†

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ABSTRACT: Ultraviolet damage endonuclease (UVDE) is a 68.7 kDa DNA repair enzyme of *Schizosaccharomyces pombe* that recognizes cyclobutane pyrimidine dimers (CPDs) and 6−4 photoproducts (6−4 PPs). UVDE is thought to initiate the first step in an alternative excision repair pathway for removal of UV light-induced DNA damage. We have overexpressed Δ228-UVDE, an active truncated form of UVDE, and have purified this protein to apparent homogeneity. We have characterized purified Δ228-UVDE with respect to its physical properties, divalent cation requirements, and kinetic parameters on oligodeoxynucleotide substrates containing a single CPD. DNA strand cleavage analysis indicates that both full-length UVDE and Δ228-UVDE incise the CPD-containing strand immediately 5′ to the lesion. These results provide further insight into the UVDE-mediated alternative excision repair pathway.

Exposure of cells to UV light results in the formation of two major classes of bipyrimidine DNA photoproducts, cyclobutane pyrimidine dimers (CPDs)[1] and 6−4 photoproducts (6−4 PPs) (*1*). CPDs and 6−4 PPs, if left unrepaired, are potentially cytotoxic and mutagenic, and organisms have evolved several different pathways for removing these lesions from cellular DNA (*1*, *2*). These include direct reversal and various excision repair pathways which can be highly specific or nonspecific for CPDs and 6−4 PPs. For example, DNA photolyases specific for either CPDs or 6−4 PPs have been found in a variety of species and restore the photoproduct bases back to their original undamaged states (*3*−*5*). Excision repair has been traditionally divided into either base excision repair (BER) or nucleotide excision repair (NER) pathways, which are mediated by separate sets of proteins but which both are comprised of DNA incision, lesion removal, gap filling, and ligation reactions (*6*, *7*). BER *N*-glycosylase/AP lyases specific for CPDs cleave the N-glycosidic bond of the CPD 5′ pyrimidine and then subsequently cleave the phosphodiester backbone at the abasic site via a β-lyase mechanism, and have been found in several species, including T4 phage-infected *Escherichia coli*, *Micrococcus luteus*, and *Saccharomyces cerevisiae* (*8*−*10*). NER is a widely distributed, lesion-nonspecific repair pathway which orchestrates DNA damage removal via a dual incision reaction upstream and downstream from the damage site, releasing an oligonucleotide containing the damage, and subsequent gap filling and ligation reactions (*7*).

Recently, we (*11*−*13*) and others (*14*−*17*) have described an alternative excision repair pathway initiated by a direct acting nuclease which recognizes and cleaves DNA containing CPDs or 6−4 PPs immediately 5′ to the photoproduct site. The initiating enzyme has been termed UV damage endonuclease (UVDE); however, the substrate specificity of the protein has not been rigorously established. Homologues of UVDE have been found in *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Bacillus subtilis*, and it can be expected that additional species will be found to possess UVDE-like enzymes (*15*−*17*). The UVDE genes from these three species have been cloned and sequenced and confer increased UV resistance when introduced into UV-sensitive strains of *E. coli*, *S. cerevisiae*, and human cells (*15*, *17*). To date, there have been no reports on the enzymatic properties of purified UVDE, and it appears that at least in some expression systems, the *Sz. pombe* protein is unstable and difficult to characterize (*17*). We report here the overexpression, purification to apparent homogeneity, and enzymatic characterization of Δ228-UVDE, a stable, active truncated version of *Sz. pombe* UVDE. The availability of this protein in large quantities should facilitate subsequent biochemical and structural studies of the UVDE-mediated alternative excision repair pathway.

MATERIALS AND METHODS

Strains and Vectors. The *E. coli* strain Top10 was used for subcloning and plasmid propagation. The *S. cerevisiae* strain DY150 (Clontech) was used for protein expression. The *S. cerevisiae* expression vector pYEX4T-1 was obtained from Clontech.

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[1] Abbreviations: BER, base excision repair; CPD, cyclobutane pyrimidine dimer; FL, full-length; GST, glutathione *S*-transferase; NER, nucleotide excision repair; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; 6−4 PP, 6−4 photoproduct; UVDE, ultraviolet damage endonuclease; Δ228-UVDE, UVDE truncation product with the first 228 N-terminal amino acids deleted.

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UVDE Gene Cloning, Expression, and Protein Purification. The full-length UVDE gene (*uvde*) coding region was initially amplified from a cDNA library (ATCC) by PCR, using the sense primer 5′-TGAGGATCCAATCGTTTTCATTTTTTAATGCTTAGG-3′ and the antisense primer 5′-GGCCATGGTTATTTTTCATCCTC-3′. To produce a truncated form of UVDE, a second sense primer was designed so that it would amplify the *uvde* coding region corresponding to the 366 C-terminal amino acids of the UVDE protein. In this case, the sense primer 5′-AATGGGATCCGATGATCATGCTCCACGA-3′ and the antisense primer 5′-GGGATCCTTATTTTTCATCCTCTTCTAC-3′ were used. The amplified UVDE gene coding fragments were cloned into the *Bam*HI and *Sma*I restriction sites of pYEX 4T-1. The Δ228-UVDE gene coding fragments were cloned into the *Bam*HI restriction site of pYEX4T-1. In the pYEX4T-1 vector, the coding region of both the proteins is expressed in-frame with a glutathione *S*-transferase (GST) leader sequence to generate a fusion protein of GST linked to the N terminus of UVDE which is under the control of the *CUP1* promoter (*18*). The subcloned plasmids were checked for orientation by restriction analysis and were then transformed into *S. cerevisiae* DY150 cells, using the alkali cation method previously described (*19*). A single positive clone was picked and grown at 30 °C until mid log phase. Cultures in mid log phase were induced with 0.5 mM $CuSO_4$. Cells (500 mL) were harvested 2 h after induction and lysed with glass beads in 50 mM Tris (pH 7.5), 100 mM EDTA, 50 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol in the presence of 0.01 mg/mL pepstatin, 3 μM leupeptin, 14.5 mM benzamidine, and 0.037 mg/mL aprotinin. The cell lysate was then dialyzed overnight in buffer without EDTA. The whole cell homogenate was separated into soluble and insoluble fractions by centrifugation at 45000*g* for 20 min. The soluble proteins (120 mg) were applied to a 2 mL glutathione−Sepharose affinity column. All purification steps were carried out at 4 °C and are similar to the strategies employed for the purification of other types of GST-tagged proteins (*18*, *20*). Unbound proteins were removed by washing with 30 mL of phosphate-buffered saline (pH 7.4), 5 mM EDTA, and 0.15 mM PMSF. GST−Δ228-UVDE was eluted (100−200 μL fractions) with 10 mM glutathione in 50 mM Tris (pH 7.4) or cleaved on the column with an excess of thrombin as previously described (*20*) to generate Δ228-UVDE without the GST tag. SDS−PAGE analysis of flow-through, wash, elution, and thrombin cleavage fractions indicated the extent of purification or GST tag removal via thrombin cleavage (Figure 1A,B). Pooled fractions containing near-homogeneous or homogeneous GST−Δ228-UVDE yielded approximately 1.5 mg of protein per 500 mL of *S. cerevisiae* cell culture.

UVDE Activity Assay. The UVDE substrate used in this study is a synthetic duplex oligodeoxynucleotide (CPD-30mer) with a cis-syn CPD (T∧T) positioned near the middle of the sequence and was a gift from J.-S. Taylor (Washington University, St. Louis, MO). The sequence of the CPD-containing strand is 5′-CATGCCTGCACGAAT∧TAAGCAATTCGTAAT-3′. The CPD-30mer was 5′ end-labeled with [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) using polynucleotide kinase (*21*). For UVDE reactions with end-labeled CPD-30mer, approximately 10 fmol of 5′ end-labeled CPD-30-mer was incubated with 5−100 ng of Δ228-UVDE or GST−Δ228-UVDE, in 200 mM Hepes (pH 6.5), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 150 mM NaCl for 15 min at 37 °C (10−20 μL reaction volume). The reaction products were analyzed on 20% denaturing (7 M urea) polyacrylamide gels (DNA sequencing gels) as previously described (*22*). The DNA species corresponding to the uncleaved CPD-30mer and cleavage product (14mer) were analyzed and quantified by phosphorimager analysis (Molecular Dynamics model 445SI) and autoradiography.

Establishment of Optimal Reaction Conditions. The optimal reaction conditions for UVDE cleavage of CPD-30mer were established by varying the NaCl concentration or divalent cation ($MnCl_2$ and $MgCl_2$) concentration or by varying the pH of the reaction buffer in the reaction. The buffers (20 mM at the indicated pH range) were as follows: sodium citrate (pH 3−6), Hepes-KOH (pH 6.5−8), and sodium carbonate (pH 9−10.6). The optimum temperature required for enzyme activity was determined by preincubating the enzyme and the substrate in the reaction buffer at a specific temperature for 10 min prior to mixing UVDE and CPD-30mer. The reaction was stopped by phenol/chloroform/isoamyl alcohol extraction, and the reaction products were analyzed on DNA sequencing gels as described above. From these experiments, the following standard reaction conditions were established: 20 mM Hepes (pH 6.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM $MnCl_2$ at 30 °C.

Kinetic Assays. Enzyme reactions were carried out with 5 nM Δ228-UVDE or 11.5 nM GST−Δ228-UVDE in 20 mM Hepes (pH 6.5) in 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 100 mM NaCl. Concentrations of CPD-30mer that was 5′ end-labeled were varied from 25 to 250 nM in a final reaction volume of 15 μL for 0−3 min at 37 °C. Initial enzyme velocities (V_i) were measured for each substrate concentration as nanomoles of product formed per second. The apparent K_m, V_{max}, and turnover number (K_{cat}) were determined from Lineweaver−Burk plots of averaged data (±standard deviations) from three independent experiments.

RESULTS

Expression and Purification of UVDE. Full-length, recombinant UVDE (FL-UVDE) and a truncated form of the enzyme Δ228-UVDE, which is missing the first 228 amino acids on the amino-terminal end, were successfully cloned and expressed in a *S. cerevisiae* GST fusion protein expression system. The rationale for expressing this truncated form of UVDE was based on an earlier report of the ability of a plasmid containing the corresponding truncated gene to confer UV resistance in a DNA repair-deficient strain of *E. coli* (*17*). Yeast DY150 cells expressing the GST-truncated UVDE fusion protein (GST−Δ228-UVDE) were lysed, and the soluble cell lysate was subjected to glutathione affinity column chromatography (Materials and Methods). SDS−PAGE analysis of various column fractions revealed several containing a near-homogeneous or homogeneous preparation of GST−Δ228-UVDE (Figure 1A, lanes 4−8). The eluted GST−Δ228-UVDE has an electrophoretic mobility consistent with its predicted size (approximately 68.7 kDa). Pooled homogeneous fractions of GST−Δ228-UVDE were used in subsequent experiments. Both crude and purified GST−Δ228-UVDE exhibited a high level of activity on an oligodeoxynucleotide substrate (CPD-30mer) contain-

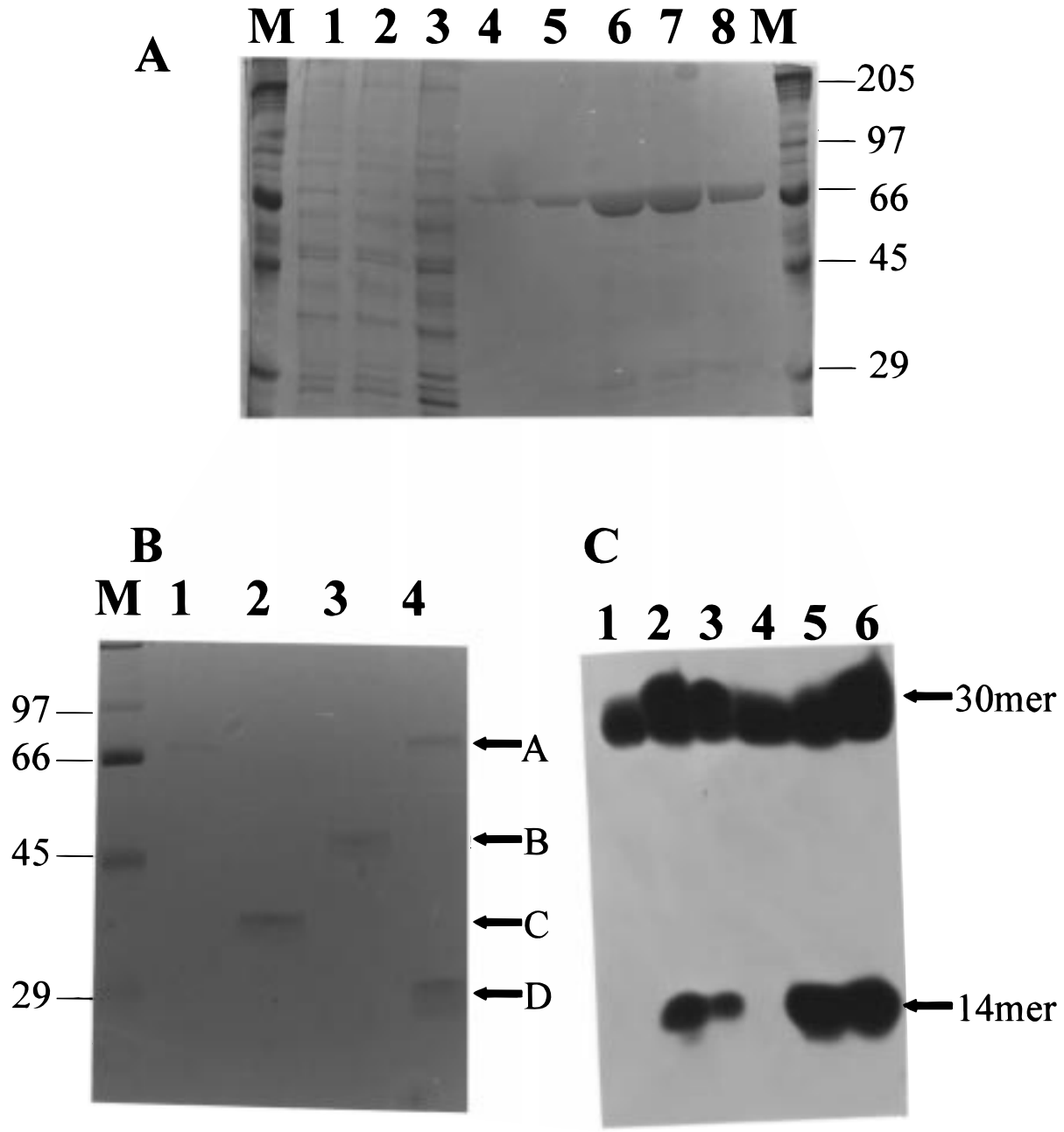

FIGURE 1: Purification and activity of GST−Δ228-UVDE and Δ228-UVDE. GST−Δ228-UVDE and Δ228-UVDE from overexpressing *S. cerevisiae* DY150 cells were purified by affinity chromatography on glutathione−Sepharose columns as described in Materials and Methods. (A) Purification of GST−Δ228-UVDE. Proteins were visualized on a silver-stained 12% SDS−polyacrylamide gel: lanes M, protein molecular mass markers (sizes indicated on the right); lane 1, 0.5 μg of soluble protein (column load) from crude extract of *S. cerevisiae* overexpressing cells; lane 2, 0.5 μg of unbound protein from affinity column flow-through; lane 3, 1.0 μg of unbound protein from column wash fractions; and lanes 4−8, equal volume (20 μL) loads of column fractions from affinity column-bound proteins eluted with glutathione corresponding to 5, 15, 65, 55, and 35 ng of protein, respectively. (B) SDS−PAGE analysis (sliver-stained 12% gel) of proteins following reapplication of GST−Δ228-UVDE onto glutathione−Sepharose and on-column thrombin cleavage to remove the GST tag: lane M, protein molecular mass markers (sizes indicated on left); lane 1, 100 ng of GST−Δ228-UVDE (column load); lane 2, 250 ng of thrombin reference marker; lane 3, 250 ng of Δ228-UVDE eluted from the column following thrombin cleavage; and lane 4, 400 ng (total protein) of GST−Δ228-UVDE and GST remaining bound to the affinity column following thrombin cleavage and elution with glutathione (Materials and Methods). Arrows indicate the positions of GST−Δ228-UVDE (A, 68.7 kDa), Δ228-UVDE (B, 41.2 kDa), thrombin (C, 37 kDa), and GST (D, 27.5 kDa). (C) Activities of GST−Δ228-UVDE and Δ228-UVDE preparations on CPD-30mer. CPD-30mer was incubated with the following preparations of UVDE: crude extract of overexpressing cells containing vector alone (lane 1), GST−Δ228-UVDE (lane 2), FL-UVDE (lane 3), affinity-purified GST alone (lane 4), affinity-purified GST−Δ228-UVDE (lane 5), and affinity-purified Δ228-UVDE (lane 6). Oligonucleotide cleavage products (14mer) corresponding to UVDE-mediated DNA strand scission of CPD-30mer immediately 5′ to the CPD site were analyzed on DNA sequencing gels and subjected to autoradiography and phosphorimager analysis as described in Materials and Methods.

ing a single cis-syn cyclobutane pyrimidine dimer embedded near the center of the sequence (Figure 1C). GST−Δ228-UVDE-mediated DNA strand scission occurred immediately 5′ to the CPD as expected and is identical to the action of native UVDE purified from *Sz. pombe* cells on the same substrate (not shown). Similar affinity purification of GST-tagged FL-UVDE (GST−FL-UVDE) from overexpressing cells resulted in a preparation that rapidly lost activity (not

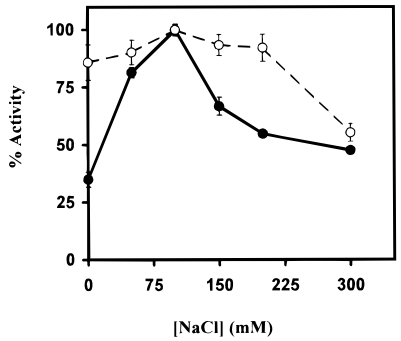

FIGURE 2: Effect of salt concentration on UVDE activity. DNA strand scission assays on end-labeled CPD-30mer were carried out with 150 ng of affinity-purified GST−Δ228-UVDE (○) or 40 ng of affinity-purified Δ228-UVDE (●) at pH 7.5 and various concentrations of NaCl under otherwise standard reaction conditions for 20 min (Materials and Methods). The extent of DNA strand scission was determined from phosphorimager analysis of gels. Enzyme activity is expressed as a percentage of CPD-30mer cleaved relative to that observed at 100 mM NaCl (defined as 100%).

shown), although crude preparations of GST−FL-UVDE were active and somewhat more stable (Figure 1C). In-column thrombin cleavage of GST−Δ228-UVDE resulted in removal of the GST tag (27.5 kDa) and the generation of Δ228-UVDE (41.2 kDa) which exhibited activity on CPD-30mer (Figure 1B,C). The properties of purified Δ228-UVDE were also compared to those of GST−Δ228-UVDE in several characterization experiments. Crude extracts of cells expressing the GST vector alone as well as affinity-purified GST alone exhibited no activity against CPD-30mer. Purified GST−Δ228-UVDE and Δ228-UVDE are quite stable when they are stored at −80 °C in 10% glycerol for a period of at least 6 months with no substantial loss of activity (not shown). Furthermore, these preparations are resistant to several rounds of freezing and thawing. The characterization studies described below represent the first report of the properties of a purified, stable form of UVDE.

Salt, pH, and Temperature Effects. The effects of monovalent salt concentration on the activity of both homogeneous GST−Δ228-UVDE and Δ228-UVDE were compared. Both forms of UVDE exhibited a relatively high level of activity over a broad NaCl concentration range (50−300 mM) with an optimum around 100 mM (Figure 2). A similar result was obtained for crude preparations of GST−FL-UVDE (not shown). A series of experiments with different types and concentrations of divalent metal cations indicated optimal cleavage of CPD-30mer in the presence of 10 mM $MgCl_2$ and 1 mM $MnCl_2$ (not shown). The pH optimum for GST−Δ228-UVDE and Δ228-UVDE was determined over the pH range of 3−10 (Figure 3). Both purified UVDE types showed an optimum at pH 6.0−6.5 with activity sharply declining on either side of this range, indicating that the GST tag probably does not affect the folding and activity of the protein. A similar result was also obtained for crude extracts containing GST−Δ228-UVDE and Δ228-UVDE (not shown). At pH 9, a second, small peak of increased activity was observed for GST−Δ228-UVDE which may be an effect due to the presence of the GST tag. The calculated p*I* values for GST−Δ228-UVDE and Δ228-UVDE are 6.8 and 7.5, respectively. It appears that UVDE exhibits optimal activity in an environment which provides an overall net positive charge.

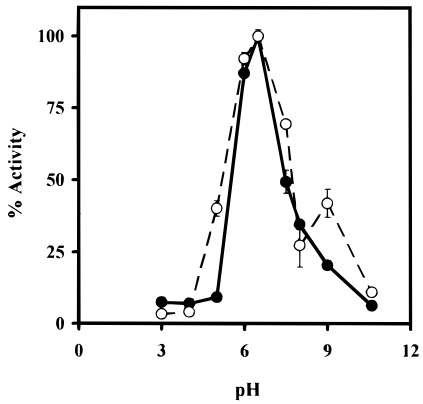

FIGURE 3: Effect of pH on UVDE activity. DNA strand scission assays on end-labeled CPD-30mer were carried out with 150 ng of affinity-purified GST−Δ228-UVDE (○) or 40 ng of affinity-purified Δ228-UVDE (●) under various pH conditions at otherwise standard reaction conditions for 20 min (Materials and Methods). The extent of DNA strand scission was determined from phosphorimager analysis of gels, and enzyme activity is expressed as a percentage of CPD-30mer cleaved relative to that observed at pH 6.5 (defined as 100%).

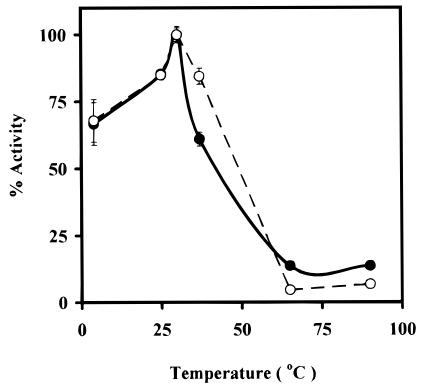

FIGURE 4: Temperature dependence of UVDE activity. DNA strand scission assays on end-labeled CPD-30mer were carried out with 150 ng of affinity-purified GST−Δ228-UVDE (○) or 40 ng of affinity-purified Δ228-UVDE (●) at the indicated temperatures under otherwise standard reaction conditions for 20 min (Materials and Methods). The extent of DNA strand scission was determined from phosphorimager analysis of gels, and enzyme activity is expressed as a percentage of CPD-30mer cleaved relative to that observed at 30 °C (defined as 100%).

Under optimal pH, salt, and divalent cation conditions, both GST−Δ228-UVDE and Δ228-UVDE were found to exhibit a temperature optimum at 30 °C, a finding which may reflect the optimal growth temperature of *Sz. pombe* (Figure 4). At 37 °C, GST−Δ228-UVDE and Δ228-UVDE activities decreased to approximately 85 and 60%, respectively, and at higher temperatures (above 65 °C), both versions of UVDE showed a significant decrease in activity. Whether the difference in activity between GST−Δ228-UVDE and Δ228-UVDE observed at 37 °C is due to a slightly increased heat stability from the presence of the N-terminal GST tag is unknown.

Kinetic Parameters. The kinetic parameters for homogeneous GST−Δ228-UVDE and Δ228-UVDE were determined using the CPD-30mer substrate. Figure 5 shows the Michaelis−Menten kinetics and Lineweaver−Burk plots for the CPD-30mer cleavage reactions with Δ228-UVDE. The apparent K_m for CPD-30mer was calculated to be 49.1 ± 7.9 nM for GST−Δ228-UVDE and 74.9 ± 3.6 nM for Δ228-

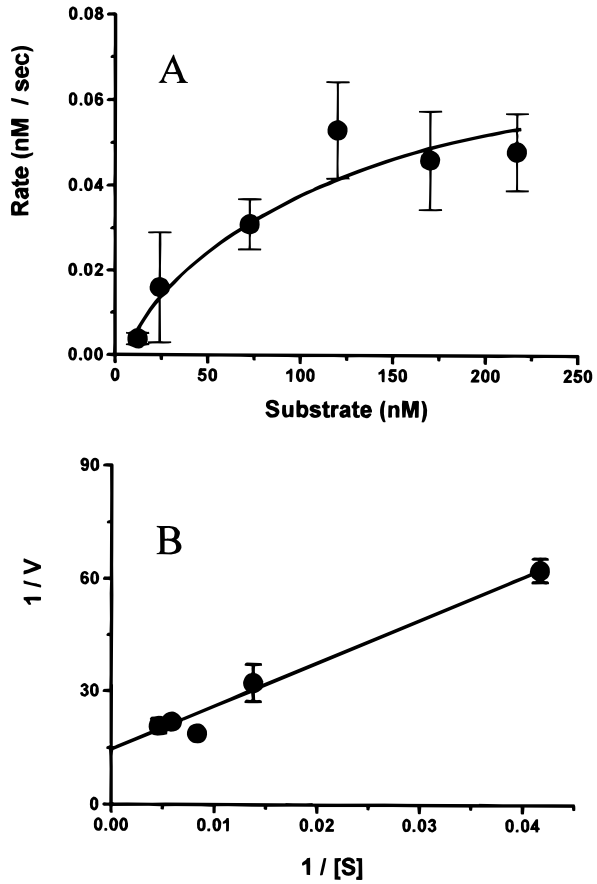

FIGURE 5: Kinetic analysis of CPD-30mer cleavage by purified Δ228-UVDE. Δ228-UVDE (5 nM) was reacted with increasing amounts of 5′ end-labeled CPD-30mer and analyzed for DNA strand scission as described in Materials and Methods. (A) Plot of reaction rate (Rate) vs substrate concentration using the mean ± standard deviation from three separate experiments. The curve is the best fit of the averaged data to the Michaelis−Menten equation. (B) Lineweaver−Burk plot of the kinetic data.

UVDE. The V_{max} values (nanomoles per minute) were found to be 2.4 ± 0.13 and 3.9 ± 0.12 for GST−Δ228-UVDE and Δ228-UVDE, respectively. The turnover numbers (K_{cat}) were 0.21 ± 0.01 min^{-1} for GST−Δ228-UVDE and 0.9 ± 0.03 min^{-1} for Δ228-UVDE. Since both types of UVDE are truncated, modified versions of the enzyme purified from a *S. cerevisiae* expression system, the kinetic parameters obtained may or may not reflect the true values of the full-length, native UVDE expressed in *Sz. pombe*.

DISCUSSION

The results of these studies provide the first report of the properties of homogeneous preparations of UVDE. Our initial attempts to purify FL-UVDE generated from the *S. cerevisiae* expression system resulted in obtaining preparations which were unstable and which formed insoluble aggregates when they were concentrated. Other groups have encountered significant difficulties during the course of expression and purification of UVDE from bacterial systems, and only partial purification of an unstable enzyme could be achieved (*17*). Taken together, this information suggests that a region contained within the 228 N-terminal amino acids of UVDE confers an instability reflecting perhaps an involvement in the regulation of UVDE levels in *Sz. pombe*. The presence of the GST tag on purified GST−Δ228-UVDE has little apparent negative effect on the activity and properties of the enzyme compared to those of purified Δ228-UVDE or crude FL-UVDE. If anything, the presence of the GST tag confers somewhat greater stability to the enzyme with respect to the effects of high salt concentrations, high pH, and elevated temperatures compared to untagged Δ228-UVDE.

UVDE initiates the first step in an alternative type excision repair pathway for the removal of bipyrimidine UV photoproducts in *Sz. pombe* as well as several other species (*15*, *17*). The putative subsequent steps following a UVDE-mediated incision adjacent to the 5′ pyrimidine of the dimer include strand displacement and resynthesis by a DNA polymerase, removal of the displaced, damage-containing strand by a flap endonuclease (FEN-1 type) such as *Sz. pombe* Rad2p, and sealing of the nicked, repaired strand with DNA ligase (*13*). A recent report has provided evidence that Rad2p is involved in UVDE-mediated repair of UV damage (*16*). We speculate that the biochemical steps after UVDE-mediated DNA strand scission may be similar to the DNA polymerase δ branch (*23*) of the base excision repair pathway which requires DNA polymerase δ (or ϵ), RF-C, PCNA, FEN-1, and DNA ligase (*24*). The availability of purified UVDE should facilitate future structural studies of the protein and biochemical studies of this excision repair pathway as well as investigations of the substrate specificity of this enzyme. Several studies have demonstrated UVDE recognition of CPDs and 6−4 PPs, but this does not rule out the possibility that other types of DNA modifications are substrates. Such studies are currently underway in our laboratory.

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