Mutation of Tryptophan 128 in T4 Endonuclease V Does Not Affect Glycosylase or Abasic Site Lyase Activity†

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ABSTRACT: Mutation of various residues within the carboxy-terminal 11 amino acids of endonuclease V, an enzyme made up of 138 amino acids that initiates the repair of cyclobutane pyrimidine dimers in DNA, has demonstrated the importance of this region in dimer-specific binding. In a previous study, substitution of a serine residue for tryptophan 128 resulted in a protein with decreased abasic site lyase activity without a concomitant decrease in DNA glycosylase activity [Nakabeppu, Y., et al. (1982) *J. Biol. Chem. 257*, 2556–2562]. To assess the importance of the tryptophan at position 128, six mutants were constructed by site-directed mutagenesis, including W128Y, W128V, W128I, W128G, W128S, and W128T. Upon characterization, these six mutants were found qualitatively to complement the repair deficiency of ultraviolet (UV) light irradiated *Escherichia coli* cells (*recA*$^-$, *uvrA*$^-$) to levels comparable to that of wild-type endonuclease V. The activities of the mutant proteins were characterized using UV-irradiated plasmid DNA and oligonucleotides containing either a site-specific cyclobutane pyrimidine dimer or an abasic site. In all cases, the six mutants displayed glycosylase and abasic site lyase activities comparable to those of wild-type endonuclease V, indicating that Trp-128 is not crucial for dimer-specific binding or catalysis.

The *denV* gene of bacteriophage T4 encodes endonuclease V, a well-characterized DNA repair enzyme that cleaves DNA at the site of UV-induced[1] pyrimidine dimers. This 16-kDa enzyme locates pyrimidine dimers by a salt-dependent one-dimensional diffusion along double-stranded DNA (Gruskin & Lloyd, 1986, 1988; Dowd & Lloyd, 1989a,b). Upon binding its substrate, the enzyme cleaves the *N*-glycosyl bond between the base and sugar of the 5′ pyrimidine within the dimer (Seawell et al., 1980; McMillan et al., 1981). The phosphodiester bond between the two dimerized pyrimidines is subsequently broken, leaving a 3′ α,β-unsaturated aldehyde and a 5′ phosphate (Weiss & Grossman, 1987). Endonuclease V can also cleave DNA 3′ to an abasic (AP) site via a β-elimination mechanism (Manoharan et al., 1988; Mazumder & Gerlt, 1989). Recent studies in this laboratory have demonstrated that the αNH_2-terminus of endonuclease V is involved in catalysis (Schrock & Lloyd, 1991, 1993) and that the reaction proceeds through an imino intermediate involving the N-terminus of the enzyme [Dodson et al., 1993; reviewed in Latham and Lloyd (1994)].

Studies with mutants of endonuclease V have revealed the importance of the C-terminal region, encompassing residues 128–137, in dimer-specific binding. Mutation of Lys-130 to histidine or glycine resulted in proteins with reduced dimer-specific binding (Recinos & Lloyd, 1988). Furthermore, conversion of Tyr-129 and Tyr-131 both to asparagine produced enzymes with no detectable pyrimidine dimer specific binding or *N*-glycosylase activity yet with wild-type AP (apurinic/apyrimidinic) lyase activity (Stump & Lloyd, 1988).

Although contained within the region implicated in pyrimidine dimer specific binding, Trp-128 seems to play a different role. Experiments on the mutant T4 phage *uvs*-13, isolated by van Minderhout et al. (1974) and sequenced by Valerie et al. (1984), yielded interesting results. The *uvs*-13 mutant contains an amber mutation in place of the Trp-128 codon within the *denV* gene. When UV-irradiated *uvs*-13 mutant T4 phage were used to infect suppressor-free (su$^-$) *Escherichia coli* cells, the phage possessed no enhanced UV survival over that of *denV*$^-$ T4 phage or T2 bacteriophage. However, when the *uvs*-13 phage were plated on suppressor-positive (su$^+$) *E. coli* that inserted a serine residue at the amber codon at position 128, the UV survival was found to approach wild-type (*denV*$^+$) levels (van Minderhout et al., 1974). When purified from su$^+$ *E. coli* and assayed in vitro, endonuclease V containing the W128S mutation exhibited near wild-type *N*-glycosylase activity, yet lacked AP lyase activity (Nakabeppu et al., 1982). In another report, mutation of W128S by site-directed mutagenesis yielded enzyme with only 3% glycosylase and 1.5% nicking activity as compared to wild type (Ishida et al., 1990). Interestingly a W128A mutant analyzed in the same study had no detectable activity on dimer-containing oligonucleotides.

In this study, site-directed mutagenesis of codon 128 within the *denV* gene encoding endonuclease V was performed in order to better understand the role of Trp-128. Six mutants were constructed, purified, and analyzed including W128Y, W128V, W128I, W128G, W128T, and W128S. One aromatic residue (Tyr) and two bulky hydrophobic residues (Val and Ile) were chosen to replace Trp-128 to determine whether or not an aromatic group or other hydrophobic amino acid side chain was required at this position for full enzymatic activity. Because previous studies had shown W128S and W128A mutants to have altered activities in some cases,

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[1] Abbreviations: UV, ultraviolet; AP, apurinic/apyrimidinic; RF, replicative form; EDTA, ethylenediaminetetraacetic acid; CS 49mer, *cis-syn* thymine dimer adducted 49mer.

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W128G, W128S, and W128T mutants were created. These last three mutants were designed to test the ability of small(Gly) and polar (Ser and Thr) residue substitutions for Trp-128 to modify the catalytic activities of endonuclease V.

EXPERIMENTAL PROCEDURES

Oligonucleotide Site-Directed Mutagenesis of denV. The *denV* gene, encoding endonuclease V, and the transcription terminator sequences were previously constructed behind the λ O_LP_R promoter in M13mp18 (Recinos & Lloyd, 1986; Recinos et al., 1986). Single-stranded M13 DNA was isolated from phage propagated in either *E. coli* UT481 (C. Lark, University of Utah) or, for uracil-containing M13 DNA, CJ236 (*ung*⁻, *dut*⁻; C. Joyce, Yale University). Mutagenic oligonucleotides were designed from the published *denV* sequence (Radany et al., 1984; Valerie et al., 1984) to alter the amino acid at position 128. Mutation of Trp-128 (TGG) to Tyr (TAC), Val (GTT), Ile (ATT), Gly (GGT), Ser (AGT), and Thr (ACG) was accomplished using oligonucleotides ranging in length from 21 to 33 nucleotides. The primers were annealed to the M13 template and extended (Zoller & Smith, 1983), and the resulting mismatch-containing plasmids were transformed into either UT481 or the mismatch repair deficient *E. coli* strain NK7085 (*MutS*⁻; P. Modrich, Duke University). Plaques containing the desired mutations were selected by differential hybridization using the ^{32}P-end-labeled mutagenic oligonucleotides as probes (Benton & Davis, 1977; Recinos & Lloyd, 1986). The M13 vectors were subsequently purified, and the mutations were confirmed by DNA sequence analyses (Sanger et al., 1977). Upon sequence analysis, the Ser-128 mutant vector was found to contain a frameshift mutation resulting from a deletion of a single nucleotide at Arg-125. This frameshift was corrected by a second round of oligonucleotide site-directed mutagenesis using a new mutagenic primer as already described, and the entire gene was sequenced to confirm that no other mutations were present.

Double-stranded RF M13mp18 DNA containing the desired mutations was isolated (Zoller & Smith, 1983) and then restricted with *Cla*I to release the *denV* gene insert. The purified *denV* fragment was then subcloned into the *E. coli* expression vector pGX2608 (Genex Corp.) at the unique *Cla*I site (Recinos & Lloyd, 1986). These plasmids were transformed into UT481 *E. coli*, and colonies were selected by ampicillin resistance. The correct insert orientation was confirmed by diagnostic *Eco*RI and *Cla*I restriction digestion (Recinos & Lloyd, 1986). Plasmids containing the mutant *denV* gene in the correct orientation were then transformed into the repair-deficient *E. coli* AB2480 (*uvrA*⁻, *recA*⁻; A. Ganesan, Stanford University).

Preparation of Mutant Enzymes. The mutant endonuclease V proteins were expressed in *E. coli* strain AB2480 utilizing the λ O_LP_R hybrid promoter within the pGX2608 constructs. The cells were grown at 30 °C for 16 h in Luria–Bertani (LB) broth media supplemented with 100 μg/mL ampicillin. Cells (10 L) were pelleted by centrifugation at 4000*g* and resuspended in 1 L of cold buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 mM KCl, and 10% (v/v) ethylene glycol. The resuspended cells were then disrupted by sonication, and the cellular debris was removed by centrifugation at 10000*g*. All mutant proteins were subsequently passed over a single-stranded DNA-agarose column and, after thorough washing of the column, eluted with a high-salt buffer consisting of 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1.5 M KCl, and 10% (v/v) ethylene glycol. Fractions containing endonuclease V were identified by SDS–polyacrylamide gel electrophoresis and immunoblot analysis (Prince et al., 1991; Schrock & Lloyd, 1993) and then combined. $(NH_4)_2SO_4$ was slowly added to the pooled fractions until the concentration approached 1 M, and the solution was loaded onto a phenyl-Sepharose column equilibrated with 25 mM NaH_2PO_4 (pH 6.8), 1 M $(NH_4)_2SO_4$, 1 mM EDTA, 100 mM KCl, and 10% (v/v) ethylene glycol. Immunoblot analysis revealed that the endonuclease V mutants, like the wild-type enzyme, did not bind to this column. The mutants were then passed over a G-100 gel filtration column equilibrated in 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM KCl, and 10% (v/v) ethylene glycol. The Ile-128 and Ser-128 mutants were further purified on a heparin–Sepharose column equilibrated in the same buffer as the G-100 column, and the proteins eluted with 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 350 mM KCl, and 10% (v/v) ethylene glycol. The final concentration of the purified mutants was determined by quantitative immunoblot analysis using various concentrations of purified wild-type endonuclease V to generate a standard curve (Gruskin & Lloyd, 1988). Wild-type endonuclease V was purified as previously described (Prince et al., 1991).

Qualitative in Vivo Assay of Mutant Enzyme Activity. E. coli strain AB2480 containing pGX2608, pGX2608-*denV*⁺, or pGX2608-*denV* mutant constructs was grown at 30 °C to stationary phase in LB media containing 100 μg/mL ampicillin. The cells were then streaked onto LB agar plates also containing 100 μg/mL ampicillin, allowed to dry, and irradiated with 254-nm light at 1 μW/cm² for increasing amounts of time. The plates were incubated at 30 °C for 24 h in the dark.

UV Damage Specific Nicking Activity of Endonuclease V Mutants on Plasmid DNA. pBR322 DNA was irradiated with 254-nm UV light to generate 20–25 pyrimidine dimers per plasmid molecule (Gruskin & Lloyd, 1986). The DNA was then diluted to 0.1 mg/mL in the enzyme reaction buffer containing 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM KCl, and 100 μg/mL bovine serum albumin (BSA). Varying concentrations of wild-type or mutant endonuclease V were added, and the reaction mixtures were incubated at 37 °C for 30 min. Reactions were terminated with an equal volume of electrophoresis loading buffer [50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 40% (w/v) sucrose, 2% (w/v) SDS, 0.025% (w/v) bromphenol blue, and 0.025% (w/v) xylene cyanol]. Supercoiled (form I), nicked circular (form II), and linear (form III) DNAs were separated by electrophoresis in 1% agarose gels. After ethidium bromide staining, the individual bands were visualized with long-wavelength UV light. The gels were photographed, and the negatives were scanned with a Microtek B/W scanner interfaced to a Macintosh IIsi computer. Alternately, images of the gels were captured directly by The Imager (Appligene) and then analyzed with the VISAGE electrophoresis gel analysis system (Millipore). Correction for the decreased binding of ethidium bromide to form I DNA was accomplished by multiplying the form I band values by 1.42 (Lloyd et al., 1978).

UV Damage Specific Glycosylase Activity of Endonuclease V Mutants on Plasmid DNA. UV-irradiated pBR322 DNA was reacted with endonuclease V as described previously. Termination of the reaction and conversion of any AP sites to single-strand nicks were accomplished by the addition of an equal volume of electrophoresis loading buffer containing 150 mM NaOH and subsequent incubation at 37 °C for 5 min. In an alternate protocol, NaOH was added to a final concentration of 50 mM to end the enzymatic reaction. These samples were incubated at 37 °C for 15 min followed by the addition of electrophoresis loading buffer. The topological

forms of DNA were separated and quantified as already described.

Thymine Dimer Specific Nicking Activity of Endonuclease V Mutants on Oligonucleotides. A 49-base oligonucleotide containing a site-specific *cis-syn* thymine dimer (CS 49mer) was generously provided by Colin Smith and John-Stephen Taylor (Washington University, St. Louis, MO) with the sequence 5′-AGCTACCATGCCTGCACGAATTAAGCAA-TTCGTAATCATGGTCATAGCT-3′ (Taylor et al., 1990; Smith & Taylor, 1993). The underlined bases indicate the position of the dimer. The CS 49mer was ^{32}P-labeled on the 5′ end with T4 polynucleotide kinase and annealed to its complementary oligonucleotide. The double-stranded CS 49mer was diluted with reaction buffer [25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM KCl, and 100 μg/mL BSA], and wild-type or mutant endonuclease V was added. Reactions were carried out at 37 °C for 30 min and terminated by either placing the reaction mixture into a dry ice–ethanol bath or treating it with 1 M piperidine and heating to 90 °C for 30 min. Piperidine treatment was performed to convert any AP sites to single-strand breaks. After drying, the pellets were resuspended in loading buffer [95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromphenol blue, 0.02% (w/v) xylene cyanol] and separated through a 15% polyacrylamide gel containing 8 M urea. Bands were visualized by autoradiography of the wet gels using Hyperfilm-MP X-ray film (Amersham).

Apyrimidinic Site Specific Nicking Activity of Endonuclease V Mutants on Oligonucleotides. A 49-base oligonucleotide containing a site-specific uracil was synthesized (Midland Research) with the sequence 5′-AGCTACCAT-GCCT-GCACGAAUTAAGCAATTCGTAATCATGGT-CATAGCT-3′. This oligonucleotide was ^{32}P-labeled on the 5′ end with T4 polynucleotide kinase, annealed to its complementary oligonucleotide, and purified through NEN-sorb cartridges (DuPont) as per the supplier's instructions. The dried oligonucleotide was resuspended in buffer containing 70 mM HEPES (pH 8.0), 1 mM EDTA, and 1 mM 2-mercaptoethanol and reacted with 0.9 unit of *E. coli* uracil DNA glycosylase (generously provided by D. Mosbaugh, Oregon State University) to produce double-stranded DNA containing a site-specific apyrimidinic (AP) site (AP 49mer). Wild-type and mutant forms of endonuclease V were reacted with the AP 49mer as described for the CS 49mer, except the reactions were terminated with loading buffer and the oligonucleotides were immediately separated by polyacrylamide gel electrophoresis.

RESULTS

Qualitative UV Survival. Wild-type endonuclease V can complement the colony-forming ability of UV-irradiated repair-deficient *E. coli* strain AB2480 (*recA*$^-$, *uvrA*$^-$). This UV complementation can be observed qualitatively with a UV streak test in which AB2480 cells harboring an expression vector, such as pGX2608, containing the *denV* gene are applied to a plate and then UV-irradiated for increasing amounts of time. This UV survival test was performed on the wild-type (W128) and all six of the mutant constructs: W128Y, W128V, W128I, W128G, W128S, and W128T. *E. coli* AB2480 containing pGX2608 without the inserted *denV* gene was used as a negative control. All of the endonuclease V mutants examined except W128T complemented the AB2480 cells to at least wild-type levels. The W128T mutant complemented the cells to near wild-type levels (Figure 1). Immunoblot analysis of crude cellular lysates of the mutant endonuclease V-producing cells indicated that all mutants were expressed to levels comparable with wild type (data not shown).

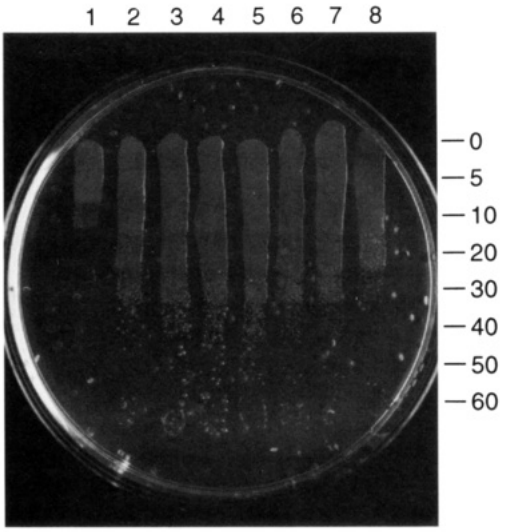

FIGURE 1: Complementation ability of endonuclease V mutants on UV-irradiated repair-deficient *E. coli* cells. *E. coli* AB2480 cells containing pGX2608 with or without the *denV* gene were grown to stationary phase, applied to an agar plate containing ampicillin, and UV-irradiated with 254-nm light at 1 μW/cm^2 for increasing amounts of time. The plates were incubated at 30 °C for 24 h in the dark. The AB2480 cells contained the constructs as follows: 1, pGX2608 (*denV*$^-$); 2, pGX2608 *denV*$^+$; 3, pGX2608 *denV* W128Y; 4, pGX2608 *denV* W128V; 5, pGX2608 *denV* W128I; 6, pGX2608 *denV* W128G; 7, pGX2608 *denV* W128S; 8, pGX2608 *denV* W128T. Times of irradiation are as indicated to the right (in seconds).

Comparison of UV Damage Specific Nicking and Glycosylase Activities. Endonuclease V functions by first locating a pyrimidine dimer by a salt-dependent linear diffusion along DNA (Lloyd et al., 1980; Gruskin & Lloyd, 1986; Ganesan et al., 1986), cleaving the glycosyl bond of the 5′ pyrimidine within the dimer, and then nicking the phosphate backbone between the dimer residues (Gordon & Haseltine, 1980; McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981). The nicking activity of endonuclease V can be monitored by using UV-irradiated supercoiled (form I) plasmid DNA (Lloyd et al., 1980). The enzyme cleaves supercoiled DNA to form a nicked circular (form II) DNA molecule, which can be separated from the form I DNA by agarose gel electrophoresis. Increasing concentrations of wild-type and mutant forms of endonuclease V were reacted with UV-irradiated pBR322 DNA in the presence of 100 mM KCl. The high-salt conditions ensured that the enzyme located pyrimidine dimers by a three-dimensional search (Gruskin & Lloyd, 1986). The topological forms of DNA were subsequently separated by agarose gel electrophoresis, and the percentages of form I and II DNAs were determined. The disappearance of form I DNA was plotted as a function of endonuclease V concentration (Figures 2a and 3a). Figure 2a displays the UV damage specific nicking ability of the proteins containing conservative mutations at codon 128 (W128Y, W128V, and W128I). Figure 3a includes data from the W128G, W128S, and W128T mutant proteins, which represent more drastic changes in the amino acid side chain. In all cases, the mutants at position 128 catalyzed an enzyme concentration-dependent decrease in the fraction of form I DNA that was comparable to the wild-type reaction.

If endonuclease V catalyzes the glycosylase cut without a subsequent phosphodiester bond scission, an abasic (AP) site will result, leaving the supercoiled structure of the plasmid intact. NaOH treatment of the endonuclease V-reacted plasmid will convert any AP sites to single-strand breaks, again yielding form II DNA, such that any glycosylase activity without subsequent lyase activity is readily observable. A serine mutant at position 128, formed by suppression of an

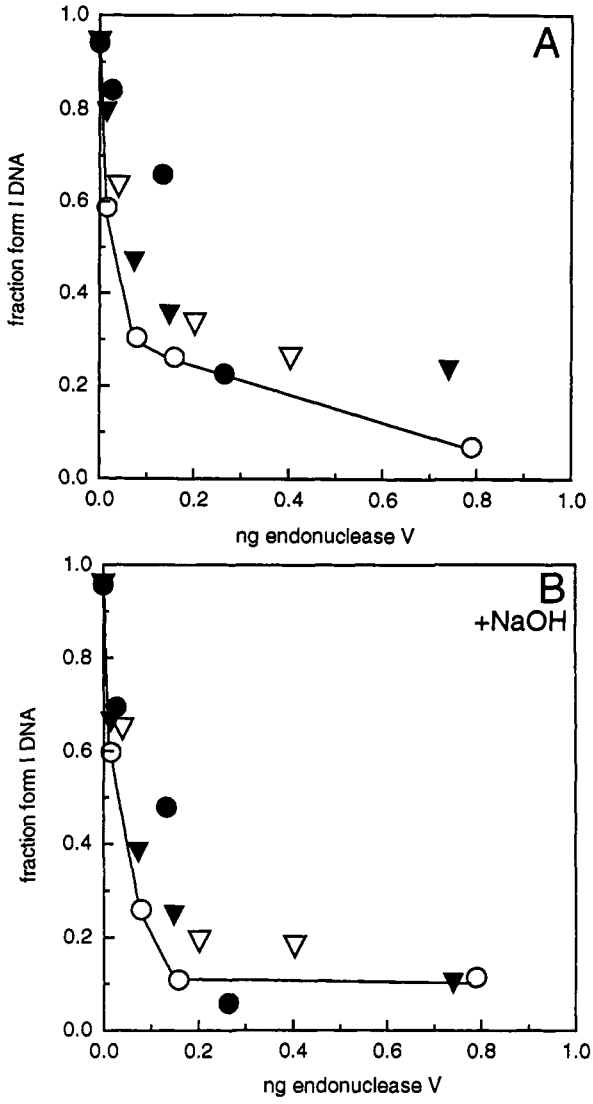

FIGURE 2: Pyrimidine dimer specific nicking and glycosylase activities of endonuclease V mutants W128Y, W128V, and W128I. Increasing amounts of wild-type or mutant endonuclease V were added to UV-irradiated pBR322 DNA. Reactions were allowed to proceed for 30 min at 37 °C. (A) Pyrimidine dimer specific nicking activity of endonuclease V mutants. Reactions were terminated with electrophoresis loading buffer containing 2% SDS, and the topological forms of DNA were separated by agarose gel electrophoresis. (B) Glycosylase activity of endonuclease V mutants. Reactions were performed as before and terminated with electrophoresis loading buffer containing 150 mM NaOH. The mixtures were then incubated at 37 °C for 5 min before agarose gel electrophoresis. The NaOH converts any AP sites to single-strand breaks, allowing glycosylase events without subsequent AP lyase events to be visualized. ○, wild-type; ●, W128Y; ▽, W128V; ▼, W128I.

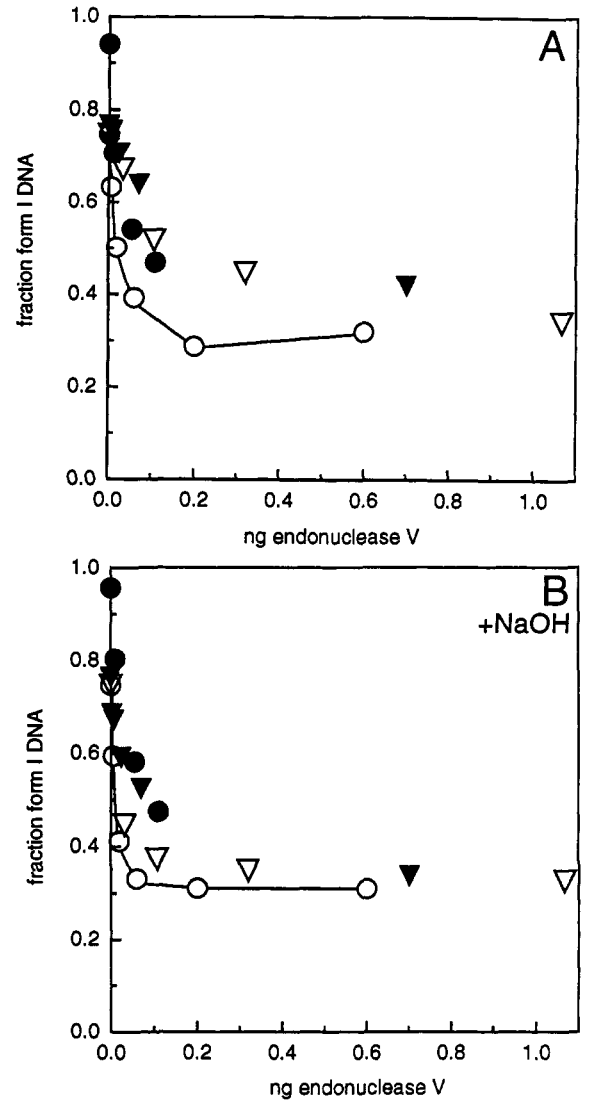

FIGURE 3: Pyrimidine dimer specific nicking and glycosylase activities of endonuclease V mutants W128G, W128S, and W128T. Reactions were performed as in Figure 2. (A) Nicking activity of endonuclease V mutants. Reactions were terminated with electrophoresis loading buffer containing 2% SDS. (B) Glycosylase activity of endonuclease V mutants. Reactions were terminated with the addition of NaOH to a final concentration of 50 mM and subsequently incubated at 37 °C for 15 min. Loading buffer was added, and the forms of DNA were separated by agarose gel electrophoresis as previously described. ○, wild-type; ●, W128G; ▽, W128S; ▼, W128T.

amber mutation at that site, was reported to possess *N*-glycosylase activity without full nicking activity (Nakabeppu et al., 1982). To examine the possibility that some of the codon 128 mutant enzymes have diminished levels of AP lyase activity, NaOH was added after the endonuclease V-catalyzed reaction to convert any AP sites to single-strand breaks (Figures 2b and 3b). Again, all six of the mutants were found to nick UV-irradiated plasmid DNA in a wild-type manner. Little difference was seen between the untreated and NaOH-treated DNAs after reaction with either wild-type or mutant endonuclease V molecules, indicating that few AP sites were left in the DNA.

Pyrimidine Dimer Specific Glycosylase versus Nicking Activities of Wild-Type and Mutant Endonuclease V Molecules on Oligonucleotides. The six mutants studied (W128Y, W128V, W128I, W128G, W128S, and W128T) all nicked UV-irradiated plasmid DNA at levels comparable to wild-type endonuclease V. These results seem to contradict the results from the analysis of the W128S mutant created by suppression of an amber codon (Nakabeppu et al., 1982). Because of these discrepancies, we decided to further analyze our mutants using oligonucleotides containing site-specific lesions. A 49-base oligonucleotide containing a site-specific *cis-syn* cyclobutane thymine dimer (CS 49mer) was previously constructed by Smith and Taylor (1993). This CS 49mer was used to further characterize the ability of mutant endonuclease V molecules to nick pyrimidine dimer containing DNA. If the enzyme successfully makes the glycosylase incision without making a subsequent AP lyase cut, an AP site will be left in the DNA. Treating the DNA with piperidine and examining the cleavage products will allow the comparison of glycosylase and full nicking activities among the different mutants. W128S and W128T were the first mutants analyzed with the CS 49mer (Figure 4). The CS 49mer was ^{32}P-labeled on the 5′ terminus and annealed to its 49mer complement. Two different concentrations of mutant enzyme were then

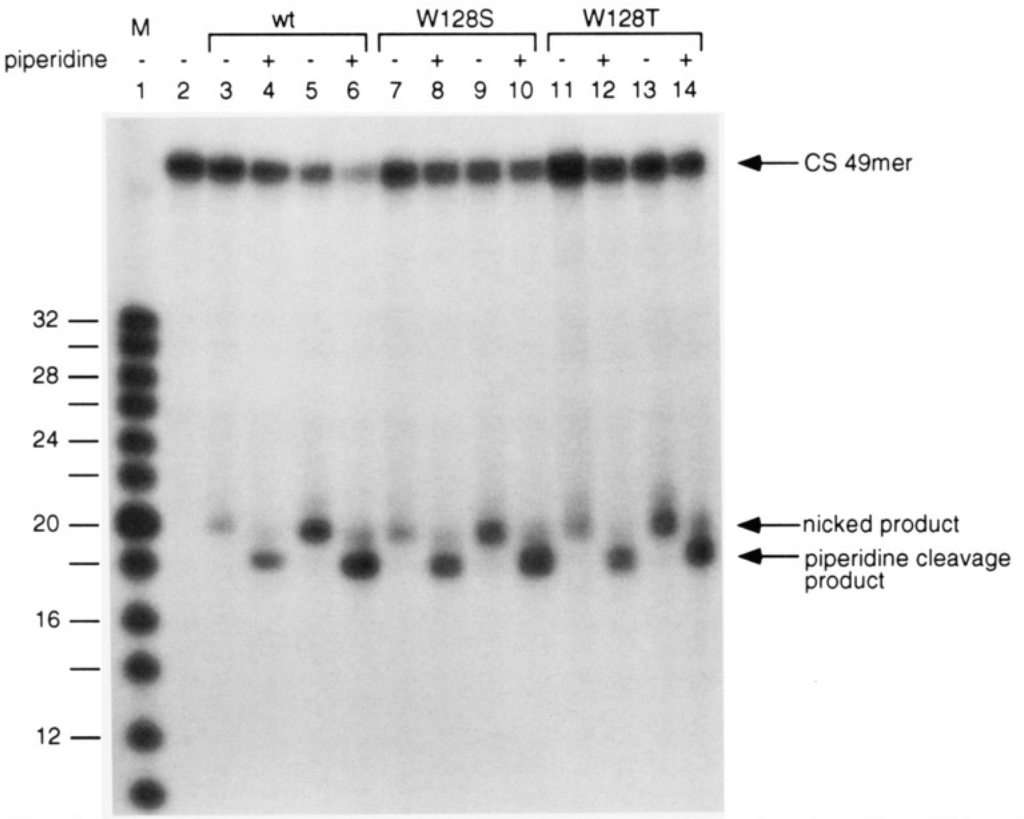

FIGURE 4: Comparison of nicking and glycosylase activities of wild-type, W128S, and W128T endonuclease V on a 49-base oligonucleotide containing a site-specific thymine dimer. A 49 base pair oligonucleotide containing a single cyclobutane thymine dimer (CS 49mer) was ^{32}P-labeled, annealed to its complementary oligonucleotide, and allowed to react with two different concentrations of endonuclease V. Half of the reactions were treated with piperidine to convert any AP sites to single-strand breaks. Lane 1, oligonucleotide markers with lengths as indicated to the left; lane 2, CS 49mer with no enzyme added; lane 3, 0.012 ng of wild type; lane 4, 0.012 ng of wild type followed by piperidine treatment; lane 5, 0.024 ng of wild type; lane 6, 0.024 ng of wild type followed by piperidine treatment; lane 7, 0.13 ng of W128S; lane 8, 0.13 ng of W128S followed by piperidine treatment; lane 9, 0.32 ng of W128S; lane 10, 0.32 ng of W128S followed by piperidine treatment; lane 11, 0.07 ng of W128T; lane 12, 0.07 ng of W128T followed by piperidine treatment; lane 13, 0.14 ng of W128T; lane 14, 0.14 ng of W128T followed by piperidine treatment.

added to the radiolabeled CS 49mer. After reaction, half of the samples were treated with piperidine to convert any remaining AP sites to DNA breaks. Electrophoresis loading buffer was added to both the untreated and piperidine-treated samples, and the products were separated by urea-containing polyacrylamide gel electrophoresis. The products of the endonuclease V reaction (Figure 4, odd lanes 3–13) exhibited an electrophoretic mobility consistent with a β-elimination mechanism (Bailly et al., 1989). Treatment of the resultant α,β-unsaturated aldehyde with piperidine caused δ-elimination, characterized by the removal of the sugar fragment, leaving a 3′ phosphate terminus, which had a faster mobility than the corresponding α,β-unsaturated aldehyde (Figure 4, even lanes 4–14). In the reactions with the lower concentrations of wild-type enzyme, there was a difference in intensity between the untreated (10% conversion of CS 49mer to product; lane 3) and piperidine-treated (32% conversion to product; lane 4) product bands, indicating that a few AP sites remained in the DNA after reaction. This intensity difference did not increase, however, in the reactions with the W128S (13% conversion to product without, and 45% conversion to product with, piperidine treatment; lanes 7 and 8) or W128T (7% conversion to product without, and 30% conversion to product with, piperidine treatement; lanes 11 and 12) mutants when compared to wild type. Therefore, as compared to wild type, the W128S and W128T mutants do not possess an impaired ability to make the phosphodiester bond scission once the glycosylase bond has been broken.

The other four mutants (W128Y, W128V, W128I, and W128G) were also assayed with the CS 49mer (Figure 5). In all cases, the mutants effectively cleaved the DNA at the thymine dimer site. Only the W128V mutant yielded a qualitatively different product: the W128V mutant reacted with the CS 49mer to produce two major nicked product bands. Less than 50% of the product had a mobility consistent with the expected 3′ α,β-unsaturated aldehyde. The identity of the second product is unclear, although the mobility appears inconsistent with a DNA fragment containing a 3′ phosphate terminus.

AP Lyase Activity of W128S and W128T Mutants on Oligonucleotides. Wild-type endonuclease V recognizes and incises AP sites to produce 3′ α,β-unsaturated aldehyde and 5′ phosphate termini (Manoharan et al., 1988; Mazumder & Gerlt, 1989; Mazumder et al., 1991). Because the W128S mutant, created by suppression of an amber mutation at codon 128, lacked AP lyase activity when assayed on dimer-containing DNA (Nakabeppu et al., 1982), we decided to examine the activity of W128S and W128T, which were created by site-directed mutagenesis, on AP-containing DNA. A 49-base oligonucleotide containing a site-specific uracil was ^{32}P-labeled, annealed to its complement, and then reacted with uracil DNA glycosylase to produce a double-stranded oligonucleotide with a site-specific AP site (AP 49mer). Piperidine treatment of the AP 49mer was used to demonstrate the complete removal of uracil, which is characterized by the complete conversion of substrate 49mer to cleaved product (Figure 6, lane 14). No cleavage was observed upon piperidine treatment of the uracil-containing oligonucleotide (data not shown). Increasing concentrations of wild-type, W128S, and W128T endonuclease V were allowed to react with the AP 49mer for 30 min. The wild-type enzyme cleaved the AP 49mer in a concentration-dependent manner to produce a

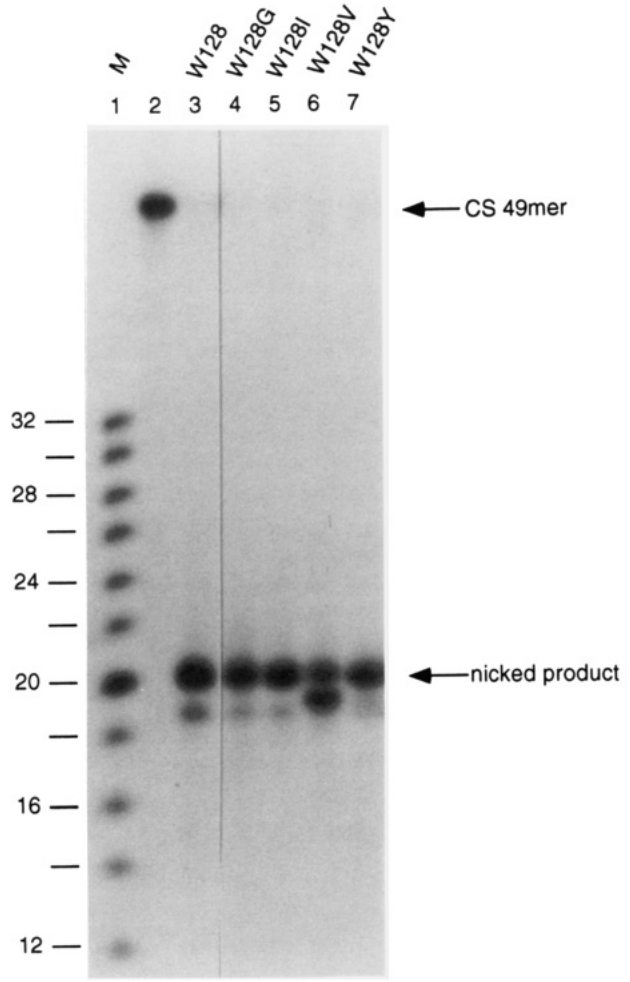

FIGURE 5: Nicking activity of mutants W128G, W128I, W128V, and W128Y on CS 49mer. The four mutants, not tested previously, were assayed for their overall ability to nick a double-stranded dimer containing 49mer oligonucleotide. The CS 49mer was labeled and reacted as in Figure 4. Lane 1, oligonucleotide markers with lengths as indicated; lane 2, CS 49mer; lane 3, wild type; lane 4, W128G; lane 5, W128I; lane 6, W128V; lane 7, W128Y.

product with the expected mobility (Figure 6, lanes 3–5). Both the W128S and W128T mutants incised the AP site in a manner consistent with the wild-type enzyme, proving there is no defect in AP lyase activity in these mutants (Figure 6, lanes 6–13). The other four mutants (W128Y, W128V, W128I, and W128G) were also found to efficiently nick the AP 49mer (data not shown).

DISCUSSION

Studies of various endonuclease V mutants have revealed the importance of the C-terminal 11 amino acids in dimer-specific binding (Recinos & Lloyd, 1988; Stump & Lloyd, 1988; Lloyd & Augustine, 1989). Mutation of residues in this region leads to enzymes which have altered abilities to locate their cyclobutane pyrimidine dimer substrates. For instance, K130H and K130G mutants exhibited reduced dimer-specific binding as compared to wild-type (Recinos & Lloyd, 1988). Similarly, the double mutant Y129N and Y131N had no measurable pyrimidine dimer specific binding capability, yet this mutant retained apurinic/apyrimidinic (AP) lyase activity, indicating that pyrimidine dimer specific binding is separable from AP site-specific binding (Stump & Lloyd, 1988). Interestingly, other C-terminal mutants display altered AP lyase activity with and without modified dimer-specific binding capabilities, including a W128S mutant that was created by the suppression of an amber mutation at codon 128. Studies with this W128S mutant demonstrated its ability to break the most 5′ glycosyl bond of a pyrimidine dimer but an inability to cleave the phosphodiester backbone after that initial glycosylase action (Nakabeppu et al., 1982). A W128S mutant created by site-directed mutagenesis, in contrast, displayed both a reduced glycosylase activity and a reduced AP lyase activity when assayed on 14mer oligonucleotides containing a single cyclobutane dimer (Ishida et al., 1990).

This study used site-directed mutagenesis of codon 128 in an attempt to better understand the role of Trp-128 in endonuclease V function. Six mutants were created, including W128Y, W128V, W128I, W128G, W128S, and W128T. The first three mutants represent conservative changes to bulky, nonpolar residues. The last three mutants represent less conservative changes to smaller (W128G) or polar (W128S and W128T) residues. Upon examination of a qualitative in vivo UV survival assay, plasmid constructs expressing all six of the endonuclease V mutants were found to complement repair-deficient AB2480 (*uvrA*$^-$, *recA*$^-$) *E. coli* to levels comparable to that of the construct expressing wild-type enzyme. The W128T construct was the only one with less than wild-type complementation, although it complemented the cells to nearly wild-type levels. There was little difference in protein accumulation among the mutants, so it does not appear that a variation in expression can account for this minor reduction in UV complementation. The W128T mutant may have a slightly diminished ability to recognize pyrimidine dimers in vivo, although no difference was seen between its activity and the wild-type enzyme's activity when assayed in vitro. The ability of all of the mutants to complement repair-deficient *E. coli* was not surprising. Even if a mutant had an impaired AP lyase activity, as in the W128S mutant studied by Nakabeppu et al. (1982), one would expect the cellular AP endonucleases to repair any AP sites remaining in the DNA.

To assess whether or not the mutants could cleave pyrimidine dimer containing DNA in vitro, the mutants were reacted with both UV-irradiated plasmid DNA and oligonucleotides containing a site-specific *cis-syn* cyclobutane thymine dimer. In all cases, the mutants cleaved dimer-containing DNA in a manner indistinguishable from wild type. These data were rather surprising, given the results of Nakabeppu et al. (1982) and Ishida et al. (1990). Nakabeppu and co-workers examined the ability of a W128S mutant, created by suppression of an amber codon, to cleave UV-irradiated T4 DNA and found that the mutant incised the glycosyl bond without subsequently cleaving the phosphodiester backbone. The truncated amber mutant, produced in a suppressor-free *E. coli* host, was found to lack both activities. In our study, the W128S mutant (and the W128Y, W128V, W128I, W128G, and W128T mutants) produced by site-directed mutagenesis was found to have both wild-type glycosylase and AP lyase activities when assayed on UV-irradiated plasmid DNA and on an oligonucleotide containing a site-specific cyclobutane thymine dimer. The W128S mutant, along with the other five mutants examined, also displayed a wild-type ability to incise oligonucleotides containing a site-specific AP site. It is possible, however, that the two studies are reconcilable. T4 DNA is modified to contain glucosylated, hydroxymethylated cytosine. The W128S mutant could exhibit an altered activity on the modified T4 DNA and not on normal DNA. Alternately, in suppression of a nonsense codon, only a fraction of the resultant peptides contain the desired amino acid. The remaining peptides are truncated at the stop codon. Truncated proteins within a preparation of otherwise normal protein could cause an alteration in activity, especially if the protein acts as a multimer. Endonuclease V has been found to exist as a dimer

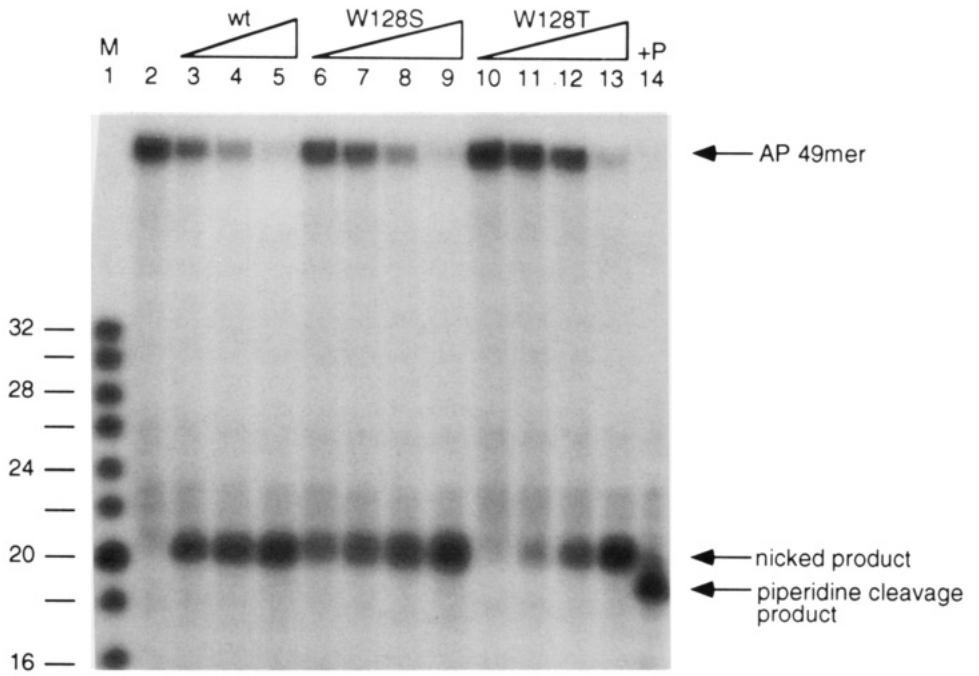

FIGURE 6: AP lyase activity of W128S and W128T endonuclease V mutants on an oligonucleotide containing a site-specific AP site. A 49-base oligonucleotide containing a single uracil residue was ^{32}P-labeled, annealed to its complementary sequence, and reacted with uracil DNA glycosylase to produce an oligonucleotide containing a site-specific AP site (AP 49mer) The AP 49mer was allowed to react with increasing concentrations of wild-type and mutant endonuclease V for 30 min at 37°C. The DNA products were then separated by urea-containing polyacrylamide gel electrophoresis. Lane 1, oligonucleotide markers; lane 2, AP 49mer (no enzyme); lane 3, 0.02 ng of wild type; lane 4, 0.06 ng of wild type; lane 5, 0.2 ng of wild type; lane 6, 0.032 ng of W128S; lane 7, 0.064 ng of W128S; lane 8, 0.13 ng of W128S; lane 9, 0.32 ng of W128S; lane 10, 0.007 ng of W128T; lane 11, 0.023 ng of W128T; lane 12, 0.07 ng of W128T; lane 13, 0.7 ng of W128T; lane 14, AP 49mer treated with piperidine.

in solution (Nickell & Lloyd, 1991), and electron microscopy has revealed that the enzyme can interact with two separate molecules of double-stranded DNA (Lloyd et al., 1987). Considering these data, it is likely that the enzyme is interacting as a dimer with DNA. If the W128S mutant preparation used in the previous study (Nakabeppu et al., 1982) contained a large amount of truncated endonuclease V, the W128S mutant could be dimerizing with truncated protein, leading to an alteration in activity. The creation of a heterodimer between the truncated protein and the otherwise silent mutant W128S could be causing the deficiency of AP lyase activity in these preparations. Testing this possibility would require the assay of a combination of truncated and wild-type endonuclease V for both glycosylase and AP lyase activities.

Another study of C-terminal region endonuclease V mutants created by site-directed mutagenesis has demonstrated a W128S mutant to have impaired glycosylase and AP lyase activities on a thymine dimer containing 14mer oligonucleotide. This mutant, however, was still able to complement repair-deficient *E. coli* after UV treatment (Ishida et al., 1990). A W128A mutant of endonuclease V was examined by the same group and found to have no ability to complement repair-deficient *E. coli* or to cleave the thymine dimer containing 14mer. Again, these results seem to contradict the data presented herein. It is unclear why the W128A mutant should have no activity, as the W128G, W128S, and W128T mutants, all representing substitutions of small amino acid residues, yielded proteins with wild-type activity in vivo and in vitro as measured on plasmid DNA and 49mer oligonucleotides. Actually, we have created the W128A mutant, and in a UV streak test, this mutant complemented repair-deficient *E. coli* cells up to wild-type endonuclease V levels (data not shown). These results clearly contradict the results of the previous study. If the W128A mutant described by Ishida and co-workers were sequestered in inclusion bodies, it would be unavailable for catalysis and thus explain the lack of complementation seen in vivo. In the in vitro analyses of the W128S and W128A mutants described by Ishida et al. (1990), the mutants were denatured with urea and then renatured before being assayed with the thymine dimer containing 14mer DNA. In this laboratory, we have not been able to successfully restore enzyme activity from denatured endonuclease V preparations. The diminished in vitro activity of the mutants described by Ishida and co-workers could be partially rationalized by a reduction in activity due to the inability to successfully renature the proteins. There are other possible explanations, however. The W128S mutant created by site-directed mutagenesis, which was found to have a wild-type ability to complement repair-deficient *E. coli* in a UV survival assay, has been previously examined in vitro only with pyrimidine dimer containing 14mer oligonucleotides (Ishida et al., 1990). It is possible that this mutant catalyzes reactions like wild type on larger DNA substrates. The mutation may only manifest a change in activity on very short oligonucleotides, since our data demonstrate this mutant to have activities indistinguishable from wild type on longer dimer containing substrates (both plasmid and 49mer DNA).

The crystal structure of endonuclease V has been solved to a resolution of 1.6 Å (Morikawa et al., 1992). In the structure, there are three α-helices which interact with a loop formed by amino acids 128–132, which are near the C-terminus. The N-terminus penetrates between two of the helices and lies approximately 20 Å from the C-terminus. Our laboratory has previously shown that the αNH_2-terminus is the catalytic residue for both glycosylase and AP lyase activities (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993). The C-terminus, on the other hand, has been shown by site-directed mutagenesis to be involved in dimer-specific binding (Recinos & Lloyd, 1988; Stump & Lloyd, 1988). It is difficult to reconcile the separation of these two important regions without allowing for the possibility that endonuclease V undergoes a conformational change upon binding either nontarget DNA or a

pyrimidine dimer site. Because endonuclease V does seem to act as a dimer, these two regions could be juxtaposed, allowing for both cyclobutane dimer recognition and catalysis by two different subunits. More likely, since the two regions important in substrate binding and catalysis are likely to simultaneously contact the DNA, the enzyme undergoes a conformational change upon interaction with DNA. Site-directed mutagenesis studies have shown the importance of a number of positively charged residues, including Arg-3 (Dowd & Lloyd, 1989a,b) and Arg-26 (Dowd & Lloyd, 1990), in the nontarget DNA scanning ability of endonuclease V. Examination of the crystal structure shows Arg-3, Arg-26, Arg-22, and Arg-117 to be near the catalytic αNH_2-terminus, and these residues are proposed to interact with nontarget DNA (Morikawa et al., 1992). The C-terminus, critical for dimer-specific binding, is also likely to be in close contact with the DNA. We postulate that, upon binding nontarget DNA, the enzyme adopts a conformation such that the C-terminus, the N-terminus, and the positively charged residues shown to be important in DNA scanning all contact the DNA. In this conformation, the C-terminus would be closer to the N-terminus, perhaps allowing the C-terminal loop to monitor the DNA for structural distortions characteristic of pyrimidine dimers. Alteration of the tryptophan at position 128 could subtly alter the ability of the enzyme to make this necessary conformational change. This alteration may only elicit a measurable effect on small oligonucleotide substrates, explaining the observed decrease in endonuclease V activity on the 14mer substrates without a decrease in activity on longer substrates.

Analysis of six endonuclease V mutants at position 128, including W128Y, W128V, W128I, W128G, W128S, and W128T, demonstrated that alteration of this residue does not affect glycosylase activity or AP lyase activity when measured on (1) UV-irradiated plasmid DNA, (2) an oligonucleotide containing a site-specific thymine dimer, and (3) an oligonucleotide containing a site-specific AP site. These results indicate that Trp-128 is not directly involved in dimer-specific binding or in catalysis.

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