# Site-Directed Mutagenesis of the T4 Endonuclease V Gene: The Role of Arginine-3 in the Target Search†

Diane R. Dowd and R. Stephen Lloyd*

*Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232*

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ABSTRACT: Endonuclease V, a pyrimidine dimer specific endonuclease in T4 bacteriophage, is able to scan DNA, recognize pyrimidine dimer photoproducts produced by exposure to ultraviolet light, and effectively incise DNA through a two-step mechanism at the damaged bases. The interaction of endonuclease V with nontarget DNA is thought to occur via electrostatic interactions between basic amino acids and the acidic phosphate DNA backbone. Arginine-3 was chosen as a potential candidate for involvement in this protein–nontarget DNA interaction and was extensively mutated to assess its role. The mutations include changes to Asp, Glu, Leu, and Lys and deleting it from the enzyme. Deletion of Arg-3 resulted in an enzyme that retained marginal levels of AP specificity, but no other detectable activity. Charge reversal to Glu-3 and Asp-3 results in proteins that exhibit AP-specific nicking and low levels of dimer-specific nicking. These enzymes are incapable of affecting cellular survival of repair-deficient *Escherichia coli* after irradiation. Mutations of Arg-3 to Lys-3 or Leu-3 also are unable to complement repair-deficient *E. coli*. However, these two proteins do exhibit a substantial level of in vitro dimer- and AP-specific nicking. The mechanism by which the Leu-3 and Lys-3 mutant enzymes locate pyrimidine dimers within a population of heavily irradiated plasmid DNA molecules appears to be significantly different from that for the wild-type enzyme. The wild-type endonuclease V processively incises all dimers on an individual plasmid prior to dissociation from that plasmid and subsequent reassociation with other plasmids, yet neither of these mutants exhibits any of the characteristics of this processive nicking activity. Rather, dimers within a population of DNA molecules appear to be encountered and incised randomly by endonuclease V Leu-3 and Lys-3, not processively. The results of these mutations suggest that the presence of a neutral or basic amino acid is necessary for substantial levels of in vitro enzymatic activity, and Arg-3 is absolutely required for the effective target search.

The means by which DNA interactive proteins recognize and locate their target sites within large domains of DNA is important for both the functional control of gene expression and the genetic integrity of the cell, yet is not well understood. There are a small number of DNA interactive proteins for which an X-ray crystal structure is known; of these, the analyses of cocrystals of proteins with target DNA sequences are available for only the restriction enzyme *Eco*RI (McClarin et al., 1986), DNase I (Oefner & Suck, 1986), λ 434 represser (Anderson et al., 1987; Aggarwal et al., 1988), λ repressor (Jordan & Pabo, 1988), and trp aporepressor (Zhang et al., 1987). Because these structures represent a static view of the final protein–target site interaction, they do not necessarily yield information as to what contacts are made between the protein and nontarget DNA sequences. To address the question of what sites within a protein are responsible for such nontarget DNA interactions as scanning or sliding, it may be necessary to cocrystallize proteins with nontarget DNA sequences and to evaluate the specific interactions. An alternative approach is to employ the technique of oligonucleotide site-directed mutagenesis. This method is used to specifically alter codons within a gene, thereby altering amino acids that are suspected to be involved in nontarget DNA interactions. The biochemical properties of those altered enzymes can then be evaluated. We have utilized the latter technique to investigate the role that a specific amino acid within a DNA repair enzyme plays in nontarget DNA interactions.

The enzyme of interest, T4 endonuclease V, is responsible for the initiation of pyrimidine dimer repair in T4-infected *Escherichia coli* (Yasuda & Sekiguchi, 1970; Friedberg & King, 1971; Simon et al., 1975). Endonuclease V has been shown to have four specific activities: (1) a salt-sensitive linear diffusion along double-stranded DNA, also referred to as scanning or sliding; (2) a pyrimidine dimer specific binding activity; (3) a DNA glycosylase activity that is specific for the 5′ glycosylic bond of a pyrimidine dimer; and (4) an apyrimidinic/apurinic (AP)[1] endonuclease activity that either can occur immediately following the breakage of the glycosylic bond of a dimer or can incise DNA at the site of a lost base (Gordon & Haseltine, 1980; Lloyd et al., 1980; Radany & Friedberg, 1980; Seawell et al., 1980; McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981; Nakabeppu et al., 1982; Ganesan et al., 1986; Gruskin & Lloyd, 1986). Endonuclease V locates its target site by a one-dimensional diffusion mechanism along nontarget DNA both in vitro (Lloyd et al., 1980; Gruskin & Lloyd, 1986; Ganesan et al., 1986) and in vivo (Gruskin & Lloyd, 1988) and processively incises pyrimidine dimers in the DNA. A similar one-dimensional walk has been observed for the *E. coli* lac repressor (Riggs et al., 1970; Berg et al., 1981, 1982; Winter

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* Author to whom correspondence should be addressed.

[1] Abbreviations: RF, replicative form; LB, luria broth; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; AP, apurinic or apyrimidinic; Tris, tris(hydroxymethyl)aminomethane.

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& von Hippel, 1981; Winter et al., 1981; Barkley, 1981), the restriction enzyme *Eco*RI (Jack et al., 1982; Langowski et al., 1983; Ehbrecht et al., 1985; Terry et al., 1985), RNA polymerase (Belinstev et al., 1980; Hannon et al., 1980; Park et al., 1982; Roe & Record, 1985; Wheeler et al., 1987; Singer & Wu, 1987, 1988), the bacteriophage λ cro protein (Kim et al., 1987), the *Bam*HI methylase (Nardone et al., 1986), and the *Bam*HI endonuclease (Nardone et al., 1986). This type of binding or association of proteins with nontarget DNA characteristically occurs through electrostatic interactions that are sensitive to the monovalent salt concentration of the solution, such that in high-salt conditions the target search becomes three-dimensional. Since sliding is electrostatic by nature, the interaction between positively charged amino acids of a protein and the negatively charged DNA phosphate backbone is most probably involved [reviewed by Lohman (1986), Ptashne (1986), and von Hippel and Berg (1989)].

A general similarity of amino acids exists between the $NH_2$-terminal portion of endonuclease V and the positively charged, flexible $NH_2$-terminal "arms" of λ repressor and the *Eco*RI restriction enzyme. These arms are known to wrap around the DNA and aid in the correct positioning of the proteins on their targets (Pabo et al., 1982; Weiss et al., 1984a,b; McClarin et al., 1986). This region of endonuclease V was mutated by oligonucleotide site-directed mutagenesis, specifically at the basic residue arginine-3, to assess the role of this amino acid in the enzymatic activities of endonuclease V.

## Materials and Methods

*Oligonucleotide Site-Directed Mutagenesis of denV.* The structural gene encoding endonuclease V, *den*V, and transcription terminator sequences were previously reconstructed behind the hybrid λ $O_LP_R$ promoter in the *E. coli* expression vector M13 bacteriophage (Recinos & Lloyd, 1986; Recinos et al., 1986) (Table I), and were later subcloned into the vector pEMBL. Single-stranded M13 DNA was prepared from phage as described (Zoller & Smith, 1983), while single-stranded pEMBL DNA was prepared as described (Dente et al., 1983). The mutagenic DNA oligonucleotides were designed from the published *den*V sequence (Radany et al., 1984; Valerie et al., 1984), synthesized, and purified as described (Lloyd et al., 1986). The sequence encoded the change from Arg-3 (CGT) to Glu-3 (GAA), Lys-3 (AAG), Asp-3 (GAT), or Leu-3 (TTA) or a deletion of Arg-3. Following the primer annealing and extension steps (Zoller & Smith, 1983), *E. coli* UT481 or JM105 was transformed with M13mp8–$O_LP_R$-*den*V (Glu-3, Lys-3, Leu-3, and ΔArg-3), while *E. coli* AB2480 (*uvr*A$^-$ *uvr*A$^-$) was transformed with pEMBL–$O_LP_R$-*den*V (Asp-3). Plaques or colonies containing the mutant constructs were selected by differential hybridization using the $^{32}P$ end labeled mutagenic oligonucleotide as the probe (Benton & Davis, 1977; Recinos & Lloyd, 1986). The vectors were subsequently purified and the mutations confirmed by DNA sequence analysis. Double-stranded mutant RF M13 was prepared (Zoller & Smith, 1983), and the mutant *den*V gene inserts were then subcloned from the M13mp8 RF recombinants into the *E. coli* expression vector pGX2608 (Recinos & Lloyd, 1986), generating pGX2608–*den*V (Glu-3, Lys-3, Leu-3, and ΔArg-3). The entire operator/promoter–*den*V (Leu-3) was subcloned because during the mutagenesis the adenine of the initial ATG was unexpectedly deleted. This deletion led to a GTG start codon which is efficiently recognized as a start translation codon in *E. coli*, and a methionine is inserted (Thach et al., 1966). *E. coli* AB2480 were transformed with the plasmids, and the desired insert orientation was confirmed by restriction analysis.

*Determination of Intracellular Accumulation of the Mutant Endonuclease V Proteins.* AB2480 *E. coli* containing the plasmid encoding the mutated *den*V gene was grown to stationary phase at 30 °C in LB medium containing 100 μg/mL ampicillin, conditions previously shown to yield maximum accumulation of the enzyme (Recinos et al., 1986). The cells were then harvested and equilibrated for cell number by optical density, and crude cell lysates were analyzed for the accumulation of anti-endonuclease V immunoreactive protein by electrophoresis through an SDS–polyacrylamide gel and subsequent Western blot analysis (Laemmli, 1970; Towbin et al., 1979; Burnette, 1981). Briefly, following SDS gel electrophoresis a sandwich of Whatman 3MM papers and nitrocellulose paper surrounding the polyacrylamide gel was placed in a Bio-Rad transblot apparatus and the proteins were transferred in 5 mM Tris-HCl (pH 8.0), 30 mM glycine, and 20% methanol at 295 mA for 16 h. The nitrocellulose sheet was blocked in 40 mL of TBS [50 mM Tris-HCl (pH 8.0), 1.25 M NaCl] and 3% gelatin for 1 h at 37 °C. The blocking solution was discarded, and a 1:1000 dilution of primary antibody (mouse polyclonal anti-endonuclease V antibody) was added to a total volume of 45 mL of TBS and 3% gelatin; the primary antibody reaction was incubated at 37 °C for 16 h. The nitrocellulose sheet was rinsed three times in TBS for 5 min each, and a 1:1000 dilution of affinity-purified rabbit anti-mouse IgG horseradish peroxidase conjugate in 40 mL of TBS and 1% gelatin was added. The secondary reaction was incubated for 3 h at 37 °C, and the nitrocellulose sheet was rinsed three times for 5 min each in TBS. A solution was made consisting of 32 mg of 4-chloro-1-naphthol dissolved in 12 mL of methanol. This was added to 60 mL of TBS. Thirty percent $H_2O_2$ (120 μL) was added to this solution and immediately dispensed onto the nitrocellulose paper. After 1 min, color development was stopped by rinsing the nitrocellulose sheet in distilled water. The nitrocellulose sheet was air-dried and stored in the dark at 22 °C.

The resulting Western blots were quantitated by scanning densitometry; the levels of endonuclease V in each sample were determined by comparison to the densitometry results for cells expressing wild-type endonuclease V. Quantitation of endonuclease V in soluble cellular extracts was performed in the same manner, comparing densitometry results with results for known quantities of purified endonuclease V run on the same blot. Extracts were made by sonication of the cells in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 10% ethylene glycol. The sonicates were then subjected to centrifugation to remove the insoluble cell debris.

Due to a low level of soluble endonuclease V Lys-3 in the cell sonicates, the concentration of the enzyme was enhanced by application to a single-stranded DNA agarose column (Higgins & Lloyd, 1987). The enzyme was eluted off the column at 360 mM KCl, and the salt concentration was subsequently reduced to 25 mM KCl by passage over an FPLC Superose 12 column.

*Survival following Ultraviolet Irradiation.* AB2480 *E. coli* (*rec*A$^-$ *uvr*A$^-$) harboring pGX2608–denV$^-$, pGX2608–denV$^+$, or mutant constructs was grown to confluence at 30 °C, diluted in growth medium, spread onto plates containing LB agar plus ampicillin (100 μg/mL), and irradiated by 254-nm UV light (Spectroline lamp Model EF-16, Spectronics Corp., New York) at 2.5 μW/cm$^2$ for increasing times. The incident dose was measured with a Spectroline UV shortwave (254 nm) meter Model DM-254 N. The plates were then incubated for 36 h at 30 °C in the dark. Survival was then measured as

Table I: Bacterial Strains, Plasmids, and Bacteriophage

| strain, plasmid, or phage | genotype or phenotype | source |
|---|---|---|
| *E. coli* | | |
| UT481 | *met thy* Δ (*lac-pro*) *hsdRBam*HI *hsd*M$^+$ *supD* Tn*10*/F′ *traD36 proAB lacI*$^q$*Z*Δ M15 | C. Lark |
| AB2480 | *uvrA6 recA13 arg*$^+$ *thr-1 leu-6 thi-1 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 sup-37* | A. Ganesen |
| plasmid | | |
| pGX2608 | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ | Genex Corp. |
| pGX2608-16 *den*V$^+$ | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ endonuclease V$^+$ | this laboratory |
| pEMBL *den*V$^+$ | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ endonuclease V$^+$ | this laboratory |
| pGX2608-113 | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ endonuclease V (Glu-3) | this study |
| pGX2608-269 | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ endonuclease V (ΔArg-3) | this study |
| pGX2608-270 | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ endonuclease V (Leu-3) | this study |
| pGX2608-350 | Ap$^r$ λ $O_LP_R$ λ $t_{4s}$ galK$^+$ endonuclease V (Lys-3) | this study |
| pEMBL-198 | Ap$^r$ λ $O_LP_R$ endonuclease V (Asp-3) | this study |
| phage | | |
| M13mp8 $O_LP_R$ *den*V | λ $O_LP_R$ endonuclease V$^+$ | this laboratory |

colony-forming ability. Each point is the average of three separate measurements.

*Dimer-Specific Nicking Activity.* [$^3$H]pBR322 was irradiated by shortwave UV light (254 nm) at 348 J/m$^2$ to generate 20–25 thymine dimers per plasmid. This was then diluted to 0.05 mg/mL in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM KCl. Soluble cellular sonicate was added to 1 μg of [$^3$H]pBR322 and incubated at 37 °C for 30 min. The reaction was stopped by addition of electrophoresis loading buffer [50% sucrose, 2% SDS, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.01% bromophenol blue]. The reaction products were subjected to electrophoresis through a 1% agarose gel, and the topological forms of DNA were visualized with ethidium bromide. DNA forms I, II, and III were excised and placed in scintillation vials with 100 μL of 1 N HCl. After the agarose was melted, 10 mL of aqueous scintillation fluid was added to each vial, and $^3$H cpm was determined by scintillation counting. These measurements were reproducible to within 15%.

*AP-Endonucleolytic Activity.* Acid-depurinated [$^3$H]-pBR322 DNA was prepared as a substrate for the enzymatic activity of endonuclease V (Lindahl & Andersson, 1972; Lindahl & Nyberg, 1972; Lloyd et al., 1978). Unirradiated pBR322 (0.1 mg/mL) in 10 mM sodium citrate (pH 4.0), 10 mM Tris-HCl (pH 8.0), and 100 mM NaCl was heated for 10 min at 63 °C. The following components were sequentially added to the reaction: an equal volume of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, $^1/_{10}$ volume of 2.5 M sodium acetate (pH 5.3), and 2.5 volumes of 95% ethanol. This solution was placed in a dry ice/ethanol bath for 1 h to stop the reaction and to precipitate the DNA. The DNA was collected by centrifugation, and the pellet was solubilized in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM KCl (0.05 mg of DNA/mL of solution). The enzymatic reactions proceeded as described for pyrimidine dimer specific nicking, using total soluble protein. The amount of form I DNA remaining in each reaction was normalized to the initial percentage of form I DNA. These measurements were reproducible to within 10%.

## Results

To evaluate the role that Arg-3 in endonuclease V may have in nontarget DNA interactions, a series of mutant *den*V genes were constructed by oligonucleotide site-directed mutagenesis. After the DNA sequence of each mutant was confirmed, the genes were subcloned into an expression plasmid and introduced into repair-deficient *E. coli* (Table I).

*Accumulation of Mutant Endonuclease V Protein in Cells.* The steady-state intracellular levels of wild-type endonuclease V and each of the mutants were compared after SDS–polyacrylamide gel electrophoresis and Western blot analysis of crude cell lysates (Figure 1). Densitometric scans of the resulting Western blot revealed that endonuclease V Asp-3 (lane 5) accumulated to approximately half that of wild type (lane 2); Lys-3 (lane 3), Glu-3 (lane 4), ΔArg-3 (lane 6), and Leu-3 (lane 7) accumulated to levels approximately equivalent to that of wild type. The amount of soluble endonuclease V was measured after sonication of the cells and subsequent removal of insoluble cellular debris. In the soluble extracts, the amount of the Lys-3 mutant is significantly less than in wild type. This could be due to improper folding of a population of the mutant, causing it to aggregate as insoluble material. The amount of soluble endonuclease V Glu-3, ΔArg-3, and Leu-3 per milligram of total soluble protein is equivalent to that obtained with the wild-type enzyme. Soluble endonuclease V Asp-3 is at levels half of the wild type.

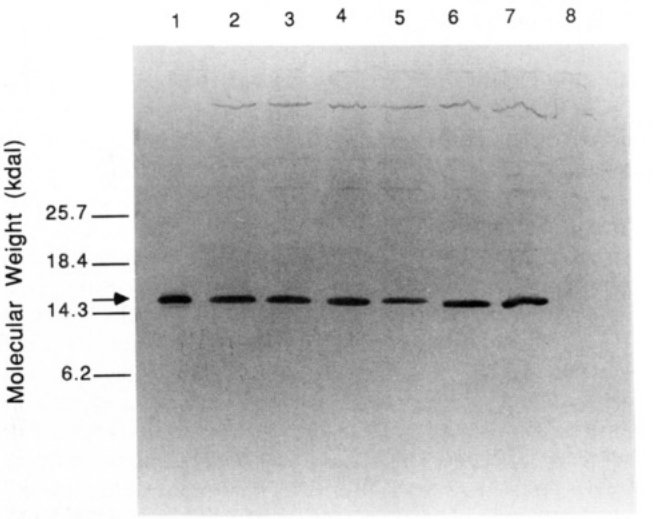


FIGURE 1: Western blot analysis of endonuclease V proteins accumulated in *E. coli* AB2480 expressing plasmid–*den*V constructs. Fifty micrograms of total cellular protein were separated through a 15% SDS–polyacrylamide gel and subjected to Western blot analysis. Mouse anti-endonuclease V polyclonal antibodies were used for detection of the 16 000-dalton protein. The endonuclease V proteins examined were purified endonuclease V (300 ng) (lane 1), Arg-3 (wild type) (lane 2), Lys-3 (lane 3), Glu-3 (lane 4), Asp-3 (lane 5), ΔArg-3 (lane 6), Leu-3 (lane 7), and cellular lysate (no endonuclease V) (lane 8). Molecular weights are as indicated.

There are a number of possibilities that could account for the slightly lower level of the Asp-3 mutant protein. The reduced accumulation could be a function of being expressed from a plasmid different from the other endonuclease V proteins. Increased protein turnover by intracellular proteolysis may contribute somewhat to the observed levels. It is also

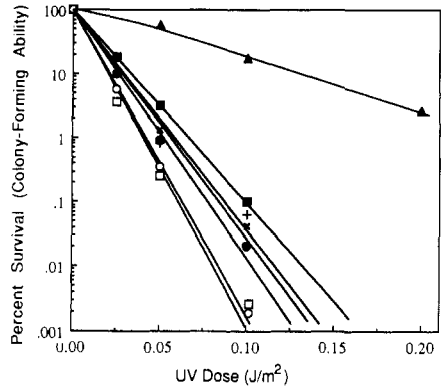


FIGURE 2: Colony-forming ability of UV-irradiated repair-deficient *E. coli* containing *denV*$^+$, *denV*$^-$, or mutant *denV* plasmid constructs. AB2480 with pGX2608-*denV*$^-$ (●); *denV*$^+$ (▲); denV-Lys-3 (■); *denV*-Glu-3 (×); *denV*-ΔArg-3 (○); *denV*-Leu-3 (□); pEMBL-*denV*-Asp-3 (+).

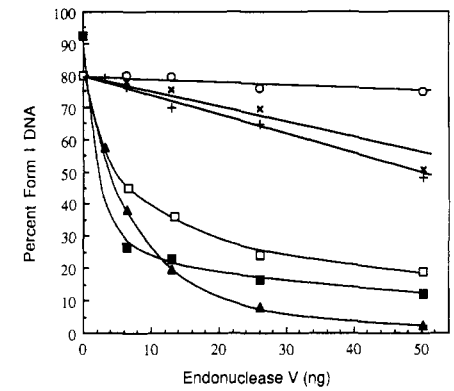


FIGURE 3: Analysis of T4 endonuclease V nicking of form I DNA containing 25 dimers per molecule. Cellular lysates containing endonuclease V were added to 1.0 μg of UV-irradiated [$^3$H]pBR322 in 20 μL of 10 mM Tris (pH 8.0), 1 mM EDTA, and 10 mM KCl. Solutions were incubated at 37 °C for 30 min. The three topological forms of DNA were separated on a 1% agarose gel. Endonuclease V Arg-3 (wild type) (▲); Lys-3 (■); Glu-3 (×); Asp-3 (+); ΔArg-3 (○); Leu-3 (□).

possible that the promoter interacts with the mutated sequence to form a secondary structure unfavorable for RNA polymerase binding, and thus the transcription of the mutant *denV* gene is inhibited.

*UV Survival As Measured by Colony-Forming Ability.* Repair-deficient *E. coli* AB2480 (*uvrA*$^-$ *recA*$^-$) that had been transformed with plasmids containing the mutant *denV* genes was irradiated with ultraviolet light for increasing times (Figure 2). Colony-forming ability of these cells following UV exposure is a measure of complete in vivo activity, since one unrepaired pyrimidine dimer is lethal to the cell. The expression of wild-type endonuclease V in these cells produces an enhanced UV survival (solid triangles); in contrast, the UV survivals of Glu-3 (×), Asp-3 (+), ΔArg-3 (open circles), Leu-3 (open squares), and Lys-3 (solid squares) are all very similar to that of the parental strain containing pGX2608–*denV*$^-$ (solid circles).

*Endonuclease V Dimer-Specific Nicking Activity.* At monovalent salt concentrations below 50 mM, wild-type endonuclease V associates with and incises irradiated form I (supercoiled) plasmid at each dimer prior to the enzyme's dissociation from that plasmid. This reaction generates either form II (nicked circular) or form III (linear) DNA. The double-strand breaks are formed if two incisions are made in close proximity and on complementary strands. This assay measures the rate of the initial cleavage event, while subsequent incisions remain undetectable unless they result in a linearized molecule. These rates can be compared directly if the incisions are random. However, in the case of wild-type endonuclease V, the incisions are not random due to the DNA scanning ability of the enzyme at low salt concentrations. When the DNA substrate contains 20–25 dimers per molecule, this assay is also a direct measure of the processive nicking activity when performed in low salt (Lloyd et al., 1980; Gruskin & Lloyd, 1986). Under processive (low salt) conditions, form III DNA accumulates in a linear manner, while distributive (high salt) conditions result in a time lag in form III DNA accumulation. Dimer-specific nicking was measured as a function of increasing endonuclease V concentration of the wild-type as well as the mutant enzymes in soluble cellular sonicates (Figure 3). Endonuclease V Glu-3 (×) and Asp-3 (+) displayed a substantial loss of nicking activity, while the deletion of Arg-3 from the enzyme (open circles) led to the abolition of all dimer-specific nicking activity. Conversely, endonuclease V Lys-3 (solid squares) and Leu-3 (open squares) exhibited nicking activity only slightly less than that seen with the wild-type enzyme (solid triangles). To further characterize the dimer-specific nicking activity of the Lys-3 and Leu-3 mutants, the reactions were monitored kinetically by using equivalent amounts of enzyme (Figure 4). Again, the experiments with both Lys-3 (solid squares) and Leu-3 (open squares) exhibited a substantial decrease in form I DNA (panel A). However, form III DNA did not accumulate in these reactions (panel C), thus suggesting that these mutant enzymes have lost their ability to scan nontarget DNA. This result was contrasted by a linear accumulation of form III DNA when the reactions were performed with wild-type endonuclease V (solid triangles).

*AP-Endonucleolytic Activity.* When endonuclease V recognizes and binds to a pyrimidine dimer, it incises the glycosylic bond of the 5′ pyrimidine of the dimer, thereby effectively producing an AP site. The enzyme then cleaves the phosphodiester bond on the 3′ side of the newly formed AP site. The glycosylase and phosphodiesterase activities are separable chemically as well as genetically; thus, DNA containing AP sites can serve as a substrate for the enzyme. To determine the AP-specific nicking activity, cellular lysates must be diluted to reduce the residual level of EDTA-resistant AP-specific nicking activity in them (Figure 5).

Although endonuclease V ΔArg-3 (open circles) did not exhibit pyrimidine dimer specific nicking, it was capable of nicking at AP sites at levels equivalent to that observed with endonuclease V Glu-3 (×). Although these activities were substantially less than that observed with wild type (solid triangles), they were slightly greater than that observed with a cellular lysate not containing endonuclease V (solid circles). The quantity of total soluble protein in the cellular lysate used to measure the residual level of AP-specific nicking is the same as that used in measuring the nicking with the Glu-3 lysate. Asp-3 (+), Leu-3 (open squares), and Lys-3 (solid squares) retained most of the AP-endonucleolytic activity of the wild-type enzyme.

## DISCUSSION

This study has focused on the interaction of endonuclease V with DNA and specifically on the role of arginine-3 in this regard. The evaluations of the site-specific alterations of the wild-type endonuclease V, which will be discussed in more detail in the following sections, have led to the following conclusions: (1) the absolute length of the $NH_2$-terminal region is critical for retaining dimer-specific nicking; (2) a neutral or basic residue is necessary for the maintenance of

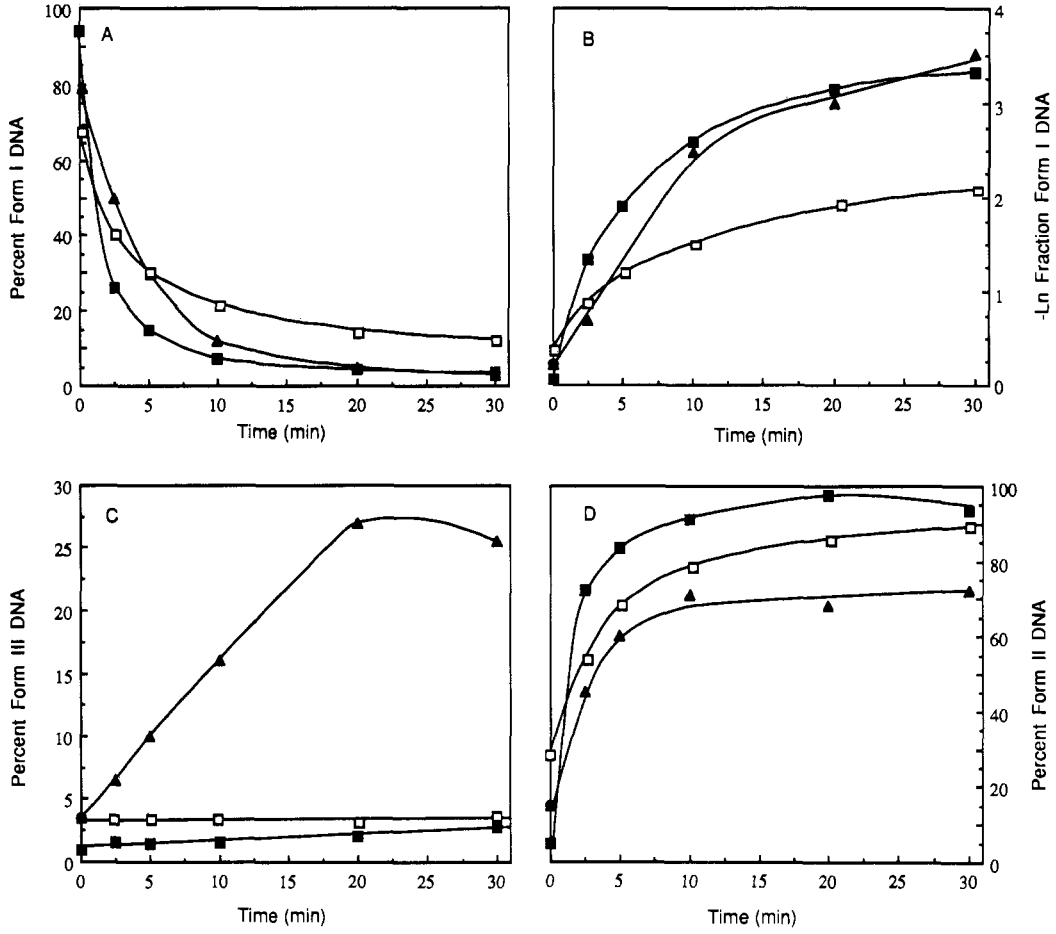


FIGURE 4: Kinetic analysis of T4 endonuclease V nicking of form I DNA containing dimers. The conditions are the same as described in Figure 3. Twenty-five nanograms of enzyme was used per point. Endonuclease V Arg-3 (wild type) (▲); Lys-3 (■); Leu-3 (□).

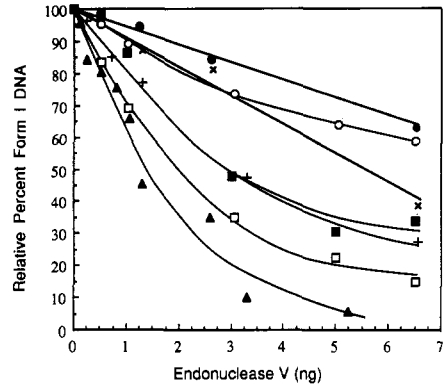


FIGURE 5: Analysis of T4 endonuclease V nicking of form I DNA containing apurinic sites. Cellular lysates containing endonuclease V were added to 1.0 μg of acid-depurinated [$^3$H]pBR322. The analysis proceeded as described in Figure 3. Total soluble protein from cells not expressing endonuclease V was analyzed at total protein levels equivalent to those used for Glu-3. Soluble protein (●); endonuclease V Arg-3 (▲); Lys-3 (■); Glu-3 (×); Asp-3 (+); ΔArg-3 (○); Leu-3 (□).

high levels of in vitro pyrimidine dimer specific enzymatic activity; (3) arginine-3 is necessary for complete in vivo activity as measured by UV resistance; and (4) arginine-3 may be responsible for an important electrostatic interaction of endonuclease V with the acidic phosphodiester backbone of DNA, and this interaction could be involved in the sliding of the enzyme along nontarget DNA. The reduced survival exhibited by all of the mutants was not due to a problem with enzyme accumulation since all enzymes were shown to accumulate in significant quantities. It is also unlikely that the enzymes were not accessible to the intracellular DNA because the mutant enzymes were soluble in a sonicated cell extract, and microscopic examination of the cells prior to sonication did not indicate the inclusion bodies (Proutz et al., 1975; Itakura et al., 1977; Goeddel et al., 1979; Cheng et al., 1981; Williams et al., 1982; Cheng, 1983) were being formed in these cells.

*Arginine-3 Deletion.* The deletion of arginine-3 resulted in a stable protein that lost virtually all in vitro activity, retaining only a very low level of AP-specific nicking activity. The loss in nicking activity was mirrored by the inability to enhance survival after UV insult. This supports the assertion that maintaining the length of the $NH_2$-terminal region is required for the dimer- and AP-specific endonucleolytic activities of endonuclease V. The loss of this residue might result in the absence of a possible electrostatic interaction as well as cause a change in the stereochemical positioning of enzymatically essential residues, thereby leading to the abolition of activity. It is also possible that this deletion significantly alters the initial steps in the proper folding of the mutant protein, thus giving rise to a virtual loss in all catalytic functions. However, since this mutant enzyme accumulates well within *E. coli* and remains soluble in sonicates, and it has been demonstrated that improperly folded proteins are subject to rapid degradation (Murakami et al., 1979; Charette et al., 1981; Parker, 1981), we consider it unlikely that the Arg-3 deletion leads to major folding alterations.

*Aspartate-3 and Glutamate-3.* The complete reversal of charge at the third residue had severe consequences on the activity of these mutants. Both enzymes retained some enzymatic activity; low levels of dimer-specific nicking were

present when reactions were performed with either enzyme, but higher enzyme concentrations were needed to achieve a loss of form I DNA greater than 20%. The reduction in the dimer-specific nicking activity was reflected in the absence of survival enhancement after UV exposure. The survival curves were indistinguishable from those exhibited by the parent repair-deficient cells not expressing endonuclease V. Again, this inability to enhance cellular survival was not due to a problem in enzyme accumulation, since both endonuclease V Glu-3 and Asp-3 accumulated to significant levels. Although the AP-specific nicking activity was decreased, the effect was not as dramatic as observed with the dimer-specific nicking. The loss in both nicking activities could be a result of abolishing a necessary electrostatic interaction between the enzyme and DNA and possibly causing a repulsion of these acidic residues with the DNA.

*Lysine-3 and Leucine-3.* The conservative alteration of Arg-3 to Lys-3 and the nonconservative change to Leu-3 had unexpected effects on enzymatic activity. Large amounts of AP- and dimer-specific nicking were exhibited by these mutants, although little to no form III DNA was detected in our nicking assays. As mentioned above, the manner in which form III DNA accumulates in a kinetic experiment is indicative of the mechanism by which endonuclease V locates and incises dimers (Gruskin & Lloyd, 1986; Dowd & Lloyd, 1989). A processive nicking mechanism, whereby endonuclease V scans DNA in a one-dimensional search for a dimer, is characterized by a linear accumulation of form III DNA over time; whereas a distributive, three-dimensional search results in a time-delayed accumulation of linear DNA (Gruskin & Lloyd, 1986). Therefore, the results obtained with endonuclease V Lys-3 and Leu-3 suggest that the processivity of the enzyme was greatly affected by these mutations. A decrease in processivity can result from either a reduction in the time spent scanning nontarget DNA or a reduction in the number of bases scanned, and we are unable to experimentally distinguish between the two possibilities. Although the change from Arg-3 to Lys-3 is a conservative one, the resulting loss in activity is not unprecedented in nucleotide-interactive proteins. Recently it was demonstrated that mutating Arg-9 to Lys-9 in pertussis toxin, a region implicated in the adenosine diphosphate ribosyltransferase activity, greatly reduced enzymatic activity (Burnette et al., 1988). Although both arginine and lysine residues are theoretically capable of interacting with phosphates in the DNA backbone, lysine-3 in endonuclease V may not be sterically competent to fulfill the role played by arginine. In contrast, although leucine has not been shown to interact with the DNA phosphate backbone, the neutral charge would not repel DNA as would aspartate or glutamate. Thus, endonuclease V Leu-3 may retain its ability to interact with dimers in an enzymatically efficient way.

The only altered in vivo characteristic of endonuclease V Lys-3 and Leu-3 is the inability to accumulate form III DNA as rapidly as the wild-type enzyme. In vivo, these mutations led to a loss in the ability to enhance the survival of repair-deficient *E. coli* after UV irradiation. It is very probable that these two characteristics are linked. Previously, it was demonstrated that a mutation in endonuclease V of Arg-3 to Gln-3 resulted in an enzyme with in vitro activities similar to those of endonuclease V Leu-3 and Lys-3. Endonuclease V Gln-3 exhibited a diminished nontarget DNA scanning capability and a subsequent loss of UV resistance (Dowd & Lloyd, 1989). A similar scenario of reduced protein–nontarget DNA interaction may be envisioned for these mutants.

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**Registry No.** L-Arg, 74-79-3; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Leu, 61-90-5; L-Lys, 56-87-1; endonuclease V, 52227-85-7.

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