Mutations in Endonuclease V That Affect both Protein–Protein Association and Target Site Location†

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ABSTRACT: A general mechanism by which proteins locate their target sites within large domains of DNA is a one-dimensional facilitated diffusion process in which the protein scans DNA in a nonspecifically bound state. An electrostatic contribution to this type of mechanism has been previously established. This study was designed to question whether other characteristics of a protein's structure might contribute to the scanning mechanism of target site location. In this regard, T4 endonuclease V was shown to establish an ionic strength dependent monomer–dimer equilibrium in solution. A protein dimer interaction site was postulated to exist along a putative α-helix containing amino acid residues 54–62. The conservative substitutions of Phe-60 → Leu-60 and Phe-59, Phe-60 → Leu-59, Leu-60 resulted in mutant enzymes which remained in the monomeric state independent of the ionic strength of the solution. The target site location mechanism of these mutants has also been altered. Under conditions where wild-type endonuclease V processively scans nontarget DNA, the target location mechanism of the monomeric mutant proteins was shifted toward a less processive search. This decrease in the processivity of the mutants was especially surprising because the nontarget DNA binding affinity was found to be significantly increased. Thus, an additional component of the endonuclease V DNA scanning mechanism appears to be the formation of a stable endonuclease V dimer complex.

Facilitated one-dimensional diffusion along nontarget DNA is a means by which several DNA-interactive proteins locate their target recognition sites. These proteins scan or slide along the contour length of the DNA in a nonspecifically bound state. This mechanism decreases the volume of solution that needs to be searched and, as a result, allows proteins to locate their recognition sites at a rate as much as 1000-fold faster than is expected for a simple three-dimensional diffusion-controlled process [for reviews, see Lohman (1986), Ptashne (1986), von Hippel and Berg (1989), and Mazur and Record (1989)]. Scanning has been observed for *Escherichia coli* lac repressor (Riggs et al., 1970); Berg et al., 1981, 1982; Winter & von Hippel, 1981; Winter et al., 1981; Barkley, 1981), *Eco*RI endonuclease (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), bacteriophage λ cro protein (Kim et al., 1987), *Bam*HI endonuclease, *Bam*HI methylase (Nardone et al., 1986), and *Micrococcus luteus* UV endonuclease (Hamilton & Lloyd, 1989).

T4 endonuclease V has also been observed to one-dimensionally scan nontarget DNA (Lloyd et al., 1980; Ganesan et al., 1986; Gruskin & Lloyd, 1986). Endonuclease V initiates the repair of ultraviolet (UV)[1]-induced cyclobutane pyrimidine dimers in T4-infected *E. coli* [reviewed by Dodson and Lloyd (1989)]. The mechanism of incision consists of the sequential action of a DNA glycosylase that cleaves the glycosyl bond of the 5′-pyrimidine of the dimer and an apyrimidinic lyase activity that cleaves the phosphodiester bond between the two pyrimidines by way of β-elimination (Haseltine et al., 1980; Radany & Friedberg, 1980; Seawell et al., 1980; Gordon & Haseltine, 1980; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981; McMillan et al., 1981; Nakabeppu et al., 1982; Manoharan et al., 1988; Mazumder et al., 1989).

The scanning or sliding mechanism of facilitated diffusion involves electrostatic interactions between the acidic phosphodiester backbone of DNA and basic amino acids on the protein surface. These interactions are sensitive to changes in in vitro ionic strength. The protein is tightly associated with nonspecific DNA at low-salt concentrations and is capable of a wide one-dimensional diffusion range. As the ionic strength increases, the dissociation rate of the protein increases, and the target location mechanism becomes a three-dimensional search (Leirmo et al., 1987). In the case of endonuclease V, the processivity of the reaction mechanism is a sensitive indicator of the one-dimensional diffusion process of scanning. At monovalent salt concentrations less than 40 mM, the enzyme slides on nontarget DNA, generating incisions at the sites of pyrimidine dimers by a processive nicking mechanism. Thus, with plasmids containing many pyrimidine dimers and a limiting enzyme concentration, the subset of plasmids which are bound by an enzyme molecule is incised at all pyrimidine dimer sites prior to enzyme dissociation. At monovalent salt concentrations above 40 mM, endonuclease V appears to no longer scan nontarget DNA. Pyrimidine dimers are incised instead by a random or distributive nicking mechanism.

Previous site-directed mutational studies of the cloned endonuclease V gene, *den*V, indicate that a highly charged region

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[1] Abbreviations: UV, ultraviolet; RF, replicative form; LB, Luria broth; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tween 20, poly(oxyethylene)sorbitan monolaurate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; AP, apurinic or apyrimidinic.

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Table I: *E. coli*, Phage, and Plasmids Used in This Study

strain, phage, or plasmid	genotype or phenotype	source
E. coli		
CJ236	dut1,ung1,thi1,relA1/pCJ105(Cm^r)	C. Joyce, Yale University
NK7085	Δ(lac pro 13) nalA mutS::Tn5	P. Modrich, Duke University
UT481	met thy Δ(lac–pro) hsdR*Bam*HI hsdM$^+$ supDTn10/F′ traD36 proAB lacIqZ Δ M15	C. Lark, University of Utah
AB2480	uvrA6 recA13	A. Ganesan, Stanford University
phage		
M13mp18-O_LP_R-*den*V		Recinos & Lloyd (1986)
M13mp18-O_LP_R-*den*V Phe-60 → Leu-60		this study
M13mp18-O_LP_R-*den*V Phe-59, Phe-60 → Leu-59, Leu-60		this study
plasmid		
pGX2608	Ap^{r}λO_LP_R λt_{4s} GalK$^+$	Genex Corp.
pGX2608-16-*den*V$^+$	Ap^{r}λO_LP_R endonuclease V$^+$λt_{4s} GalK$^+$	Recinos & Lloyd (1986)
pGX2608-16-*den*V Phe-60 → Leu-60	new 60 codon CTT	this study
pGX2608-16-*den*V Phe-59, Phe-60 → Leu-59, Leu-60	new 59 and 60 codon TTACTT	this study

of the protein electrostatically contributes to the ability of the enzyme to act processively on nontarget DNA. These results suggest that an endonuclease V–nontarget DNA interface exists along the proposed solvent-exposed face of a putative α-helix containing amino acid residues Arg-26, Ala-30, Lys-33, His-34, and Asn-37 (Dowd & Lloyd, 1990; Augustine et al., 1991; Nickell et al., 1991). In addition, Arg-3 has been demonstrated to interact with nontarget DNA (Dowd & Lloyd, 1989a,b). Thus, the electrostatic nature of nontarget DNA binding and the maintenance of the nonspecifically bound state which results in scanning have been linked to this region of endonuclease V. However, no studies have investigated the possibility that other factors may contribute to the one-dimensional diffusion mode of target location. Previous observations suggested that endonuclease V may exist and associate with DNA as a protein dimer of enzyme molecules. To further address these observations, a series of experiments were performed with wild-type endonuclease V and mutant enzymes which were altered at a putative protein dimer interaction site. The ability of endonuclease V to form a stable protein dimer, the region involved in this dimerization, and the significance of dimerization in the differential target location mechanisms utilized during pyrimidine dimer repair are described herein.

Materials and Methods

Bacteria, Phage, and Plasmids. The *E. coli* strains, M13 phage constructs, and plasmids used in this study are described in Table I.

Oligonucleotide Site-Directed Mutagenesis of denV. The structural gene encoding endonuclease V, *den*V, and transcription terminator sequences have been 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). Single-stranded uracil-containing M13 DNA was prepared from phage passaged through the *E. coli* strain CJ236 (dut$^-$ung$^-$) as described by Kunkel et al. (1987). The mutagenic DNA oligonucleotides were designed from the published *den*V sequence (Radany et al., 1984; Valerie et al., 1984) and obtained from Research Genetics, Huntsville, AL. The mutagenic oligonucleotides were designed to alter the following amino acids: Phe-60 (TTT) to Leu-60 (CTT) (21-mer with sequence 5′-CTTATCGTAAAGGAATGT-AAC-3′); Phe-59, Phe-60 (TTCTTT) to Leu-59, Leu-60 (TTACTT) (24-mer with sequence 5′-CTTATCGTAAAG-TAATGTAACATG-3′). Following the 5′-phosphorylation of the mutagenic oligonucleotides (Kunkel et al., 1987), primer annealing, and extension steps (Zoller & Smith, 1983), mismatch repair-deficient *E. coli* NK7085 (mutS$^-$) were transformed with the M13mp18-O_LP_R-*den*V mutants. Plaques containing the mutant constructs were selected by differential hybridization using the respective ^{32}P-end-labeled mutagenic oligonucleotide as the probe (Benton & Davis, 1977; Recinos & Lloyd, 1986). The mutation-containing M13 vectors were subsequently purified and the mutations confirmed by DNA sequence analysis (Sanger et al., 1977). Double-stranded mutant RF M13 DNA was prepared (Zoller & Smith, 1983) and the mutant *den*V gene inserts released by *Cla*I restriction digestion. The isolated mutant *den*V gene inserts were then subcloned into the *E. coli* expression vector pGX2608 (Recinos & Lloyd, 1986) at the unique *Cla*I site, thus generating pGX2608-*den*V-Leu-60 and pGX2608-*den*V-Leu-59,*Leu*-60. These plasmids were transformed into *E. coli* UT481. Following ampicillin selection, the desired insert orientation was confirmed by diagnostic restriction analysis (Recinos & Lloyd, 1986). Plasmids containing the mutant *den*V genes in the proper orientation were transformed into *E. coli* AB2480 (uvrA$^-$recA$^-$).

Preparation of Mutant Enzymes. Following the construction of the mutant *den*V-containing plasmids, the mutant endonuclease V proteins were expressed by using the λ O_LP_R hybrid promoter in *E. coli* AB2480 grown at 30 °C for 16 h in LB media supplemented with 100 μg/mL ampicillin. Cells (2 L) were pelleted by centrifugation at 4500*g* and resuspended in 100 mL of cold 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 mM KCl, and 10% (v/v) ethylene glycol. Cells were disrupted by sonication and the cell debris removed by centrifugation at 10000*g*. Wild-type endonuclease V and the two mutant proteins were purified by sequential use of the following chromatography steps: single-stranded DNA–agarose, G-100 gel filtration, and heparin–Sepharose (Prince et al., 1991). Of note, it was observed that both mutant proteins eluted from the heparin–Sepharose column at a salt concentration twice that necessary to remove bound wild-type endonuclease V. Following this purification scheme, all enzyme preparations were found to be free of nonspecific DNA nicking activity. The amount of the mutant endonuclease V proteins recovered by this method was determined by quantitative Western blot analyses in which pure endonuclease V was used to generate a standard curve (Gruskin & Lloyd, 1988).

Gel Filtration Chromatography Analysis. Solutions were prepared containing varying concentrations of wild-type or mutant endonuclease V in 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, and 100 mM KCl. A solution containing the molecular weight standards lysozyme (14300), trypsinogen (24000), and pepsin (34700), each at a concentration of 30 μg/mL, was also prepared in the same buffer. Aliquots of these solutions (500 μL) were injected onto a Superose-12 gel filtration column (FPLC–Pharmacia Systems) which had been preequilibrated in either 10 mM NaH_2PO_4 (pH 6.8), 1 mM

EDTA, and 10 mM KCl or 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, and 100 mM KCl. At a flow rate of 1.0 mL/min, 1-mL fractions were collected over the entire column volume. The elution pattern of the molecular weight standards was followed by absorption at 280 nm. In order to determine the elution pattern of the wild-type and mutant enzymes, fractions were assayed for the presence of immunoreactive endonuclease V. This procedure was performed at 25 °C. An aliquot of each fraction was loaded onto a nitrocellulose sheet using a Schleicher & Schuell minifold filtration dot blot manifold. The nitrocellulose sheet was blocked in 5% (v/v) horse serum in 10 mM NaH_2PO_4 (pH 7.5) and 0.9% saline (PBS) for 1 h. The blocking solution was discarded, and the sheet was rinsed 3 times, 10 min each, in PBS. The nitrocellulose sheet was incubated 16 h in a 1:25 000 dilution of primary antibody (mouse monoclonal anti-endonuclease V antibody 21:C:10) directed against amino acids 92–103. The nitrocellulose was rinsed 3 times, 10 min each, in PBS containing 0.1% (v/v) Tween 20. The nitrocellulose sheet was incubated for 30 min, according to Vector Laboratories' Vectastain ABC kit instructions, in biotinylated horse anti-mouse IgG. The sheet was rinsed 3 times, 10 min each, in PBS/Tween 20. The nitrocellulose was then incubated for 30 min in precomplexed Vectastain ABC reagent containing avidin DH and biotinylated horseradish peroxidase H. Following three 10-min PBS/Tween 20 rinses, the nitrocellulose-bound endonuclease V antigens were visualized upon the addition of a peroxidase substrate solution. This solution consisted of 32 mg of 4-chloro-1-naphthol dissolved in 12 mL of methanol, to which was added 60 mL of PBS and 120 μL of 30% H_2O_2. After full color development, the nitrocellulose sheet was rinsed in distilled water and air-dried. The relative intensity of the immunoreactive signals was determined by densitometry.

Pyrimidine Dimer Specific Nicking Activity. Form I ^{3}H-pBR322 was irradiated by 254-nm UV light at 100 μW/cm^2 for 245 s in order to generate 20–25 pyrimidine dimers per plasmid molecule (Gruskin & Lloyd, 1986). The DNA was then diluted to 0.05 mg/mL in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 10–110 mM KCl as described in the figure legends. To 1 μg of ^{3}H-pBR322 in the described solution a fixed concentration of enzyme was added and incubated at 37 °C for the indicated periods of time. The reaction was stopped by the addition of an equal volume of electrophoresis loading buffer [50% sucrose, 2% SDS, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.1% bromophenol blue]. The reaction products were subjected to electrophoresis through a 1% (w/v) agarose gel and stained with ethidium bromide, and the resulting topological forms of DNA were visualized by exposure to long-wave UV light. DNA forms I, II, and III were excised and placed in scintillation vials with 150 μL of 1 N HCl. After the agarose was melted, 10 mL of aqueous scintillation fluid was added to each vial, and the radioactivity was determined by liquid scintillation spectroscopy (Lloyd et al., 1980).

AP-Lyase Activity. Acid-depurinated ^{3}H-pBR322 DNA was prepared as a substrate for the enzymatic activity of both wild-type endonuclease V and the mutant proteins (Lindahl & Andersson, 1972; Lindahl & Nyberg, 1972; Lloyd et al., 1978). Unirradiated ^{3}H-pBR322 (0.1 mg/mL) in 10 mM sodium citrate (pH 4.0) and 100 mM NaCl was heated for 10 min at 63 °C (Lloyd et al., 1980). The following components were sequentially added to the reaction: an equal volume of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, 0.1 volume of 2.5 M sodium acetate (pH 5.3), and 2.5 volumes of 95% ice-cold ethanol. The solution was placed in a dry ice/ethanol bath for 30 min in order to stop the reaction and to precipitate the DNA. The DNA was collected by centrifugation, and the pellet was solubilized in either 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 10 mM KCl or 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 100 mM KCl to a final concentration of 0.05 mg DNA/mL of solution. To 1 μg of ^{3}H-pBR322 in the described solution varying concentrations of wild-type or mutant endonuclease V were added and incubated at 37 °C for 30 min. The reaction was terminated, and its products were analyzed as described for pyrimidine dimer specific nicking activity.

Plasmid DNA Fragment Size Distribution. A pyrimidine dimer specific nicking time course reaction was performed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 10 mM KCl as described above. Duplicate aliquots were removed from the master reaction solutions at each time point and stopped by the addition of an equal volume of electrophoresis loading buffer. One sample from each duplicate was processed by native gel electrophoresis in order to determine the relative percent of each topological form of DNA. To the duplicate sample NaOH was added to a final concentration of 100 mM. These latter samples were subjected to denaturing gel electrophoresis in 1.2% agarose gels which were cast as usual and then soaked for 16 h in 30 mM NaOH and 1 mM EDTA. Following electrophoresis at 40 V for 21 h, the gels were neutralized and the resulting length distributions of single-stranded DNA visualized by Southern blot analysis using ^{32}P nick-translated pBR322 as the probe.

Survival following Ultraviolet Irradiation. AB2480 *E. coli* (recA$^-$uvrA$^-$) harboring pGX2608, pGX2608-*den*V$^+$, or pGX2608-*den*V mutant contructs were grown to confluence at 30 °C, diluted in growth medium, spread onto LB agar plates containing ampicillin (100 μg/mL), irradiated at 2.5 μW/cm^2 for increasing periods of time, and incubated for 30 h at 30 °C in the dark. Survival is measured as colony-forming ability.

RESULTS

Experimental Rationale. Although the crystal structure of endonuclease V has not been published, Morikawa et al. (1988) have made progress in this area. In the absence of crystal information, a working model of the structural elements of T4 endonuclease V has been developed (Nickell et al., 1991). This model has served to facilitate the design and interpretation of oligonucleotide site directed mutagenesis studies which explore relevant structure–function relationships. The proposed model consists of five α-helical regions. Four of the α-helices are three-dimensionally approximated as a left-handed, antiparallel, four-α-helix bundle whose amphipathic α-helices are adjacently connected by peptide chains described only by length. The remaining α-helix lies in a region outside the four-α-helix domain which connects the adjacent bundle α-helices numbered 2 and 4. Previous electron microscopic analyses of UV-irradiated plasmid DNA incubated with endonuclease V suggest that at least one active form of endonuclease V may be a multimeric complex of enzyme molecules in association with DNA (Lloyd et al., 1987). Other high-pressure gel chromatography data indicate that enriched endonuclease V in solution is either a highly asymmetric monomer or a dimer of enzyme molecules (R. D. Schrock, III, and R. S. Lloyd, unpublished data).

T4 Endonuclease V Protein Dimer Formation. In order to further investigate the capacity of endonuclease V to form a protein dimer, a series of gel chromatography experiments were performed with pure wild-type endonuclease V in solution.

Preliminary experiments that were designed to follow the chromatographic behavior of endonuclease V under low (<40 mM) and high (>100 mM) monovalent salt concentrations revealed interesting results. It was observed that under low-salt conditions a dilute solution of endonuclease V eluted in a single immunopositive peak. However, under high-salt concentrations, a similar solution of endonuclease V separated into two immunopositive peaks.

A detailed study of this phenomenon was conducted. Duplicate samples containing 1800 ng of wild-type endonuclease V were injected onto a Pharmacia FPLC Superose-12 gel filtration column equilibrated in and eluted with 10 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, and 10 mM KCl. One set of these low-salt elution fractions was subjected to dot blot monoclonal antibody analysis in order to identify the fraction containing immunoreactive endonuclease V. All immunoreactive endonuclease V was confined to fraction 20. The KCl concentration of the fraction from the remaining duplicate set (identical with the one found to contain endonuclease V, fraction 20) was increased to 100 mM KCl. A 500-μL aliquot of this 1-mL fraction was injected onto the same column equilibrated in and eluted with 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, and 100 mM KCl. In addition, a sample containing 900 ng of wild-type endonuclease V in 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, and 100 mM KCl was injected onto the column and eluted under similar high-salt conditions. The elution pattern of endonuclease V from both high-salt chromatography injections was also determined by dot blot monoclonal antibody analysis. The pattern which resulted revealed that both the chromatographically isolated single peak and wild-type endonuclease V eluted from the column in two distinct peaks, centered around fractions 17 and 20, respectively. An estimate of the molecular weight associated with each of these endonuclease V immunopositive peaks was determined relative to the peak positions of molecular weight standards examined under similar salt concentration conditions. The calculated molecular weight of endonuclease V is 16000. Molecular weight determinations revealed that the endonuclease V found in the single peak obtained under low-salt conditions had a molecular weight of ~16000. We believe this peak represents an endonuclease V monomer. At the high salt concentration, the lower molecular weight endonuclease V peak eluted from the column at a position similar to the single monomer peak that had been obtained under low salt concentrations. Due to a significant compression of the standard curve under those high-salt conditions, this peak reflected a chromatographic molecular weight of ~10000. To ensure this peak did not represent an immunoreactive proteolytic fragment, a sample of endonuclease V in high-salt buffer was analyzed by denaturing polyacrylamide gel electrophoresis. A Coomassie stain of this gel revealed a single band with a molecular weight of ~16000. The higher molecular weight endonuclease V peak obtained under high salt concentrations was in a position reflecting a chromatographic molecular weight of 19000, nearly twice that associated with the monomer peak. From these results, we conclude that the peak associated with fraction 20 is an endonuclease V monomer while the peak associated with fraction 17 is a dimer of endonuclease V molecules. Although both of the chromatographic molecular weights at high salt were lower than expected, relative to one another they demonstrate a significant alteration in chromatographic behavior resulting solely from an ionic strength perturbation. Thus, under low-salt conditions, endonuclease V exists as a monomer in solution. However, under high-salt conditions, endonuclease V in solution assumes a monomer–dimer equilibrium.

The effect of enzyme concentration on the protein monomer–dimer equilibrium was also expolored. In data not shown, we found the elution of endonuclease V under low-salt conditions to be independent of concentration. However, in a high-salt environment, the relative concentrations of endonuclease V in the monomer and dimer state were altered by changes in protein concentration. Due to detection assay limitations, the minimum detectable protein concentration was 1×10^{-7} M. As the concentration of the enzyme was increased above the limiting concentration, the area of both peaks increased. Once a critical concentration was reached, further increase in the concentration of the enzyme resulted in an increase in the more rapidly eluting dimer peak while the monomer peak remained constant. As discussed by Cann (1970), this type of protein elution behavior is typical of the association–dissociation expected in a monomer–dimer equilibrium.

Design, Construction, and Production of Mutant Endonuclease V Protein in Cells. To further address these observations, we searched our endonuclease V structural model for potential protein dimer interaction sites. In doing so, we looked for strongly hydrophobic areas, along the perimeter of the four-α-helix bundle that were away from putative catalytic sites, and which upon interaction would be capable of forming a stable protein homodimer. Such a region was found on the putative α-helix, located outside the four-α-helix bundle domain, which contains amino acid residues 54–62. In order to identify the region of endonuclease V involved in protein dimer formation and the role that this protein dimerization plays in the location and repair of pyrimidine dimers, a series of experiments were performed with wild-type endonuclease V and mutants of endonuclease V whose *den*V genes had been altered by oligonucleotide site directed mutagenesis. These mutants (Phe-60 → Leu-60 and Phe-59, Phe-60 → Leu-59, Leu-60) were designed to have conservative amino acid substitutions in the postulated protein–protein interaction site. The wild-type enzyme and the mutants of T4 endonuclease V were expressed by utilizing the hybrid λ O_LP_R promoter in repair-deficient *E. coli* AB2480 cells. The steady-state intracellular levels of wild-type endonuclease V and each of the mutants were compared by Western blot analysis following SDS–polyacrylamide gel electrophoresis of total protein in whole cell extracts. It was observed that each of the mutant proteins accumulated intracellularly at levels that were approximately 10% of that observed with the wild-type enzyme (data not shown). Following the purification of the enzymes as described under Materials and Methods, the concentrations of the partially purified mutant enzymes were quantitated by Western blot analysis (Gruskin & Lloyd, 1988). All preparations were found to be free of nonspecific DNA nicking activity (data not shown).

Effect of Mutations on Protein Dimer Formation. The results of an earlier section clearly indicate that in a high-salt environment wild-type endonuclease V in solution exists in a monomer–dimer equilibrium. In a low-salt environment, the enzyme in solution exists solely as a monomer. The chromatographic characteristics of the two putative dimer interaction site mutants, Leu-60 and Leu-59, Leu-60, were examined under similar ionic conditions and at the same protein concentration where wild-type endonuclease V dimer formation was observed (Table II). It was noted that under low-salt conditions the two mutant proteins eluted in the monomer position as did the wild-type enzyme. Interestingly, however, at monovalent salt concentrations greater than 100 mM, only

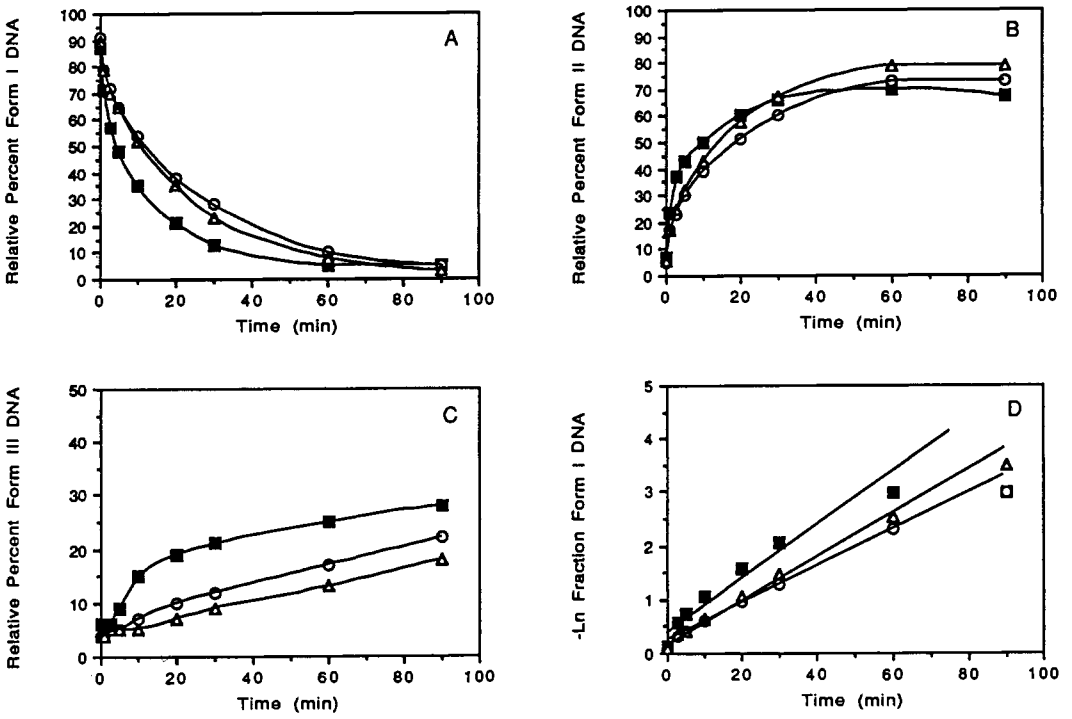

FIGURE 1: Kinetic analysis, under processive conditions, of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers. Partially purified endonuclease V was added to 1.0 μg of UV-irradiated ^{3}H-pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C, and the reaction was terminated at timed intervals. The three topological forms of DNA were separated on a 1% agarose gel, and the relative mass fraction was determined by scintillation spectroscopy. All experiments were performed in duplicate. Panel A shows the loss of form I DNA. Panel B shows the accumulation of form II DNA. Panel C shows the accumulation of form III DNA. Panel D shows the first-order rate loss of form I DNA. Endonuclease V (wild type), 1.0 ng/time point (■); Leu-60, 1.0 ng/time point (○); Leu-59, Leu-60, 1.0 ng/time point (△).

Table II: Gel Filtration Chromatography Elution Pattern of Wild-Type and Mutant Endonuclease V

	elution conditions			
	10 mM NaH_2PO_4, 1 mM EDTA, 10 mM KCl		25 mM NaH_2PO_4, 1 mM EDTA, 100 mM KCl	
sample injected	immuno-positive fraction	oligomeric state	immuno-positive fraction	oligomeric state
wild-type endonuclease V	20	monomer	17:20	dimer/monomer
Phe-60 → Leu-60	20	monomer	20	monomer
Phe-59, Phe-60 → Leu-59, Leu-60	20	monomer	19	monomer

the wild-type enzyme was found to establish an association–dissociation equilibrium resulting in the formation of both a monomer and a dimer peak. The two mutants were each found to elute as a single peak with a monomeric molecular weight. These results suggest that indeed the amino acid substitutions at positions 59 and 60 alter the protein dimerization capacity of these mutant enzymes.

Altered Protein Dimerization Ability Correlates with Altered T4 Endonuclease V Pyrimidine Dimer Specific Nicking Activity. To determine if the altered protein dimerization capacities of the two mutant endonuclease V enzymes have an effect on the mechanism of pyrimidine dimer specific nicking, kinetic analyses were performed under low- and high-salt conditions. In a pyrimidine dimer specific nicking reaction, the initial cleavage of supercoiled form I plasmid DNA produces nicked circular form II plasmid molecules. Linear form III plasmid molecules are subsequently generated when two incisions occur in close proximity on opposite DNA strands. A one-dimensional diffusion on nontarget DNA, termed scanning, results in a processive pyrimidine dimer nicking mechanism. This mechanism is characterized by the incision of all pyrimidine dimers located on a single plasmid prior to dissociation of the enzyme from that plasmid DNA molecule. This type of activity is indicated by the linear accumulation of form III plasmid DNA over time. At the other extreme, a three-dimensional target search results in a distributive pyrimidine dimer nicking mechanism. As the sites of pyrimidine dimer nicking activity become randomly distributed among the plasmid DNA molecules, form III DNA accumulation lags for a short time period until random incisions are generated in close proximity on opposite DNA strands. Under processive conditions at 10 mM KCl (Figure 1), the Leu-60 and Leu-59, Leu-60 mutants exhibited form I DNA nicking activities which proceeded at a rate only slightly less than that of the wild-type enzyme. Form III DNA accumulated linearly in the presence of wild-type endonuclease V as expected. However, a slight lag in the accumulation of form III DNA, which is indicative of a less processive nicking activity, was associated with the mutant enzymes.

To augment this possible lag in form III DNA accumulation, a similar processive kinetic analysis was performed at a reduced temperature of 25 °C (Figure 2). Under these conditions, the lag in form III accumulation observed with the mutant enzymes was clearly present at time points up to 75 min. Only at time points at which the initial wild-type linear accumulation of form III DNA had reached a plateau did the mutant enzymes begin to demonstrate a significant form III accumulation (data not shown).

In order to confirm that the mechanism of target site location had shifted from a highly processive scanning toward a more distributive reaction, denaturing agarose gel electrophoresis was employed to analyze the accumulation of single-strand DNA breaks and the size distribution of the re-

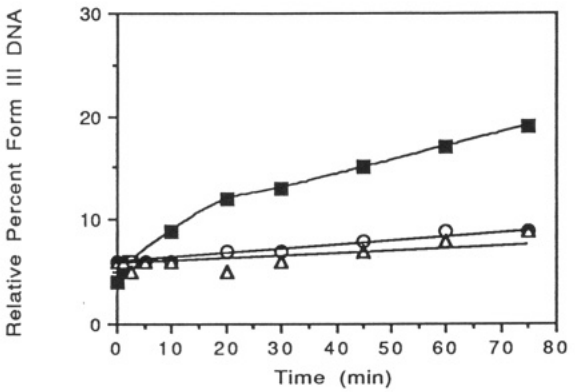

FIGURE 2: Reduced temperature kinetic analysis of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers. The conditions are the same as described in Figure 1. Solutions were incubated at 25 °C, and the reaction was terminated at timed intervals ending at 150 min. Form III accumulation over the first 75 min of the time course is shown. Endonuclease V (wild type), 1.0 ng/time point (■); Leu-60, 1.0 ng/time point (○); Leu-59, Leu-60, 1.0 ng/time point (△).

sulting DNA fragments. Gruskin and Lloyd (1988) have shown that this assay can be diagnostic of either a processive or a distributive reaction. During a fully processive nicking reaction on plasmid DNA, the majority of the substrate exists as unreacted form I DNA or as fully incised form II or form III plasmid molecules. During a fully distributive nicking reaction, there is a more rapid conversion of form I plasmids to the form II state since the production of form II DNA molecules only requires the introduction of the first break and subsequent breaks are not observed until a form III DNA is made. In addition, the DNA fragments produced at early times by using a distributive search mechanism are of a significantly larger molecular weight than those observed during a processive reaction. Kinetic analyses under conditions of low salt, identical with that described in Figure 1, were performed in duplicate. One sample from each duplicate was processed by native gel electrophoresis in order to determine the relative percent of each topological form of DNA. As shown in Figure 3, DNA samples with equivalent amounts of form I DNA remaining were analyzed by denaturing agarose gel electrophoresis. These data are in good agreement with the data on the kinetics of accumulation of form III DNA, in that the lag in form III DNA production had been interpreted to indicate that the processivity of the mutant enzymes was less than that of the wild type. The DNA fragments produced by the Leu-60 and Leu-59, Leu-60 mutants were of slightly greater size than those produced by the wild-type enzyme known to be acting processively under these conditions.

When the ionic strength conditions of the kinetic analysis are increased to 100 mM KCl, the process by which wild-type endonuclease V locates pyrimidine dimers within the UV-irradiated plasmid population in vitro is shifted to a distributive mechanism. The results of kinetic analyses conducted under distributive conditions are shown in Figure 4. It is clear that the distributive behavior of the mutant proteins was altered relative to wild-type endonuclease V. The relative rates of form I DNA loss associated with the Leu-60 and Leu-59, Leu-60 mutants were reduced by 50% and 85%, respectively. The lag in form III DNA accumulation which is indicative of a three-dimensional search was observed with both the wild-type and the mutant proteins. In fact, the delay in double-strand break formation was so great for the mutant enzymes that no form III accumulation was observed. This was true even at time points where similar low levels of form I DNA remained

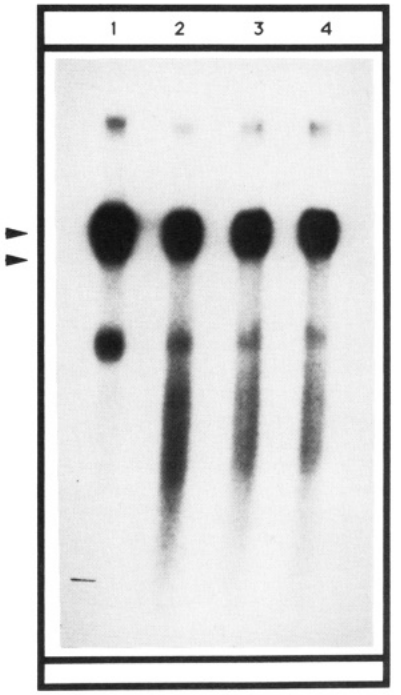

FIGURE 3: Plasmid DNA incision frequency determination. A pyrimidine dimer specific nicking time course reaction was performed at 37 °C under processive conditions. The conditions are the same as described in Figure 1. Both wild-type and mutant endonuclease V reactions containd 1 ng of enzyme/time point. Reaction products were resolved by electrophoresis through a denaturing agarose gel. The resulting single-stranded DNA fragment size distribution was visualized by Southern blot analysis using a nick-translated pBR322 probe. Lane 1, untreated pBR322; lane 2, wild-type endonuclease V, 51% form I DNA remaining; lane 3, Leu-60, 51% form I DNA remaining; lane 4, Leu-59, Leu-60, 49% form I DNA remaining. To the left of lane 1, the lower arrow reflects the migration of denatured, covalently closed, circular double-strand form I DNA. The upper arrow indicates the migration position of denatured, full-length, single-strand DNA.

in the presence of wild-type or mutant enzymes. Thus, although the mutant proteins act by a distributive mechanism of pyrimidine dimer incision, as does the wild-type enzyme, the relative specific activity of the mutants has been severely reduced.

Effect of Altered Protein Dimerization Ability on T4 Endonuclease V AP-Lyase Activity. As mentioned earlier, the endonuclease V repair mechanism is comprised of both DNA glycosylase and AP-lyase activities. These activities are separable chemically as well as genetically. Thus, DNA containing AP sites, yet lacking pyrimidine dimer sites, may serve as a substrate for the AP–lyase activity of endonuclease V. This activity results in the conversion of supercoiled form I DNA containing AP sites to nicked circular form II DNA. As shown in Figure 5, the ability of mutant enzymes to incise DNA containing AP sites was measured under low- and high-salt conditions as a function of enzyme concentration. At low ionic strength, the two mutant proteins, Leu-60 and Leu-59, Leu-60, had a relative AP-lyase activity greater than that of wild-type endonuclease V (4× and 8×, respectively). As the ionic strength of the reaction environment increased, the relative rate of the wild-type AP site incision reaction increased approximately 2-fold compared to the mutant enzymes.

T4 Endonuclease V Pyrimidine Dimer Specific Nicking Activity in the Presence of Competitor DNA—An Assay for Enzyme Binding to Nontarget DNA. The electrostatic character of the protein–nontarget DNA interaction is known to contribute to the nontarget DNA scanning ability of en-

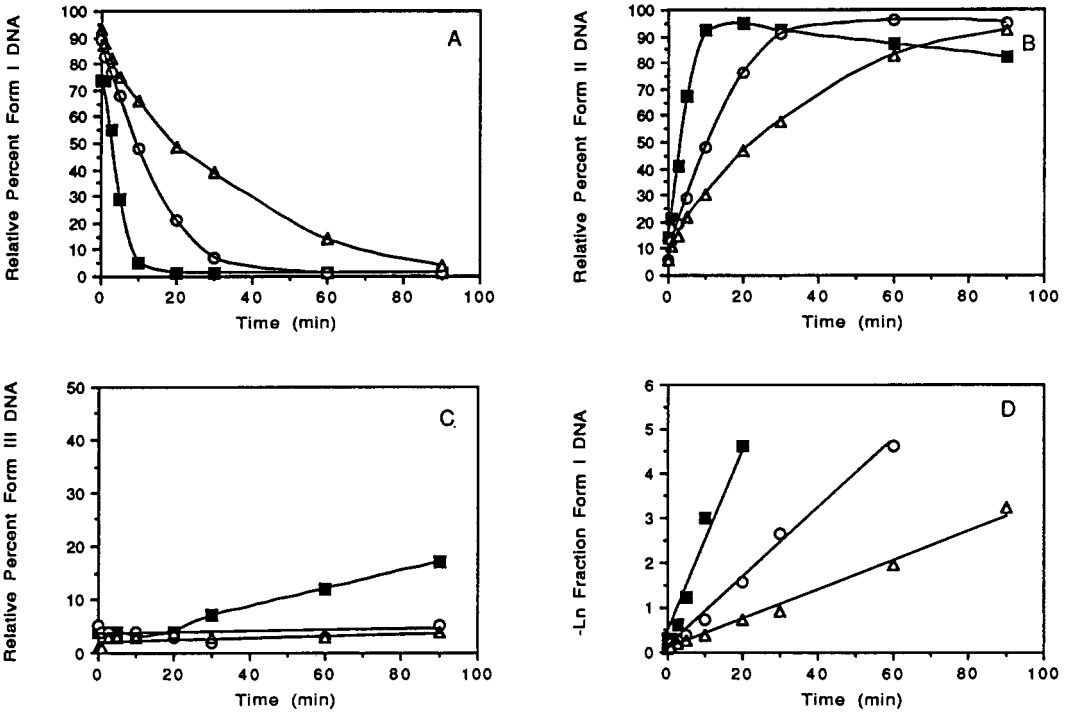

FIGURE 4: Kinetic analysis, under distributive conditions, of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers. Partially purified endonuclease V was added to 1.0 μg of UV-irradiated ^{3}H-pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C, and the reaction was terminated at the indicated time. The three topological forms of DNA were separated on a 1% agarose gel, and the relative mass fraction was determined by scintillation spectroscopy. All experiments were performed in duplicate. Panel A shows the loss of form I DNA. Panel B shows the accumulation of form II DNA. Panel C shows the accumulation of form III DNA. Panel D shows the first-order rate loss of form I DNA. Endonuclease V (wild type), 0.1 ng/time point (■); Leu-60, 0.1 ng/time point (○); Leu-59, Leu-60, 0.1 ng/time point (△).

donuclease V. This effect is demonstrated by the salt concentration dependent competition of unirradiated DNA with pyrimidine dimer containing DNA. The salt concentration at which the target site location mechanism switches from processive to distributive is approximately 40 mM (Gruskin & Lloyd, 1986; Nickell et al., 1991). At KCl concentrations where processivity is high, the loss of pyrimidine dimer containing form I plasmid DNA is inhibited by excess unirradiated DNA. Under distributive KCl concentrations (100 mM), the presence of up to a 25-fold excess of competitor DNA does not affect the random target search mechanism (Nickell et al., 1991). Even though the data presented thus far indicate that the Leu-60 and Leu-59, Leu-60 mutant proteins employ a less processive search mechanism at low-salt concentrations and a distributive search mechanism at high-salt concentration, a series of pyrimidine dimer specific compeitition assays were performed to monitor the nontarget DNA interaction of these mutants. Unexpectedly, the mutant enzymes were inhibited by the presence of unirradiated competitor DNA. Competition was evident even at salt concentrations greater than that required for wild-type endonuclease V to switch from a processive nontarget DNA interaction mechanism to a distributive mechanism. For both mutant enzymes, the KCl concentration at which the presence of excess competitor DNA resulted in a 50% inhibition of pyrimidine dimer containing form I DNA nicking was greater than the salt concentration required to achieve a 50% inhibition in the presence of wild-type enzyme (Figure 6). These results indicate that both mutant proteins have an enhanced nontarget DNA affinity. Previous interpretation of this assay has been based solely on the electrostatic contributions to nontarget DNA binding. These results which are in contrast to the earlier data suggest that processivity is dependent upon more than simple electrostatic interactions. Furthermore, the enhanced nontarget DNA affinity of these mutant enzymes may be responsible for the significantly reduced specific activity of the mutants under distributive conditions.

UV Survival of DNA Repair-Deficient Cells Expressing Mutant denV Genes. The survival of repair-deficient *E. coli* AB2480 (uvrA$^-$recA$^-$) which had been transformed with plasmids expressing the mutant *den*V genes and subsequently irradiated with UV light for increasing time periods was measured (Figure 7). The expression of wild-type endonuclease V conferred enhanced UV resistance relative to cells containing only the parental vector pGX2608. The UV resistance of cells expressing the Leu-60 and Leu-59, Leu-60 altered *den*V genes was enhanced to levels less than that of the wild-type enzyme.

Discussion

Previous studies with genetically altered forms of T4 endonuclease V have been performed in order to derive a more detailed model of the reaction process and aid in the mechanistic interpretation of the crystal structure, once it becomes available. In this regard, one area of study has focused on the one-dimensional DNA scanning mechanism of pyrimidine dimer location. In general, an increase in the lifetime of the nonspecifically bound state permits a greater sampling of DNA sequences per enzyme–DNA encounter and, thus, results in an increased rate of target location. The increased nontarget DNA affinity which results in the one-dimensional scanning of nontarget DNA and the processive incision of pyrimidine dimers has been shown to have a significant electrostatic component. The current study was designed to explore the contribution of other factors, such as stable protein dimer formation, to the endonuclease V DNA scanning mechanism.

Gel filtration chromatography experiments demonstrate that wild-type enodnuclease V establishes a salt-dependent protein

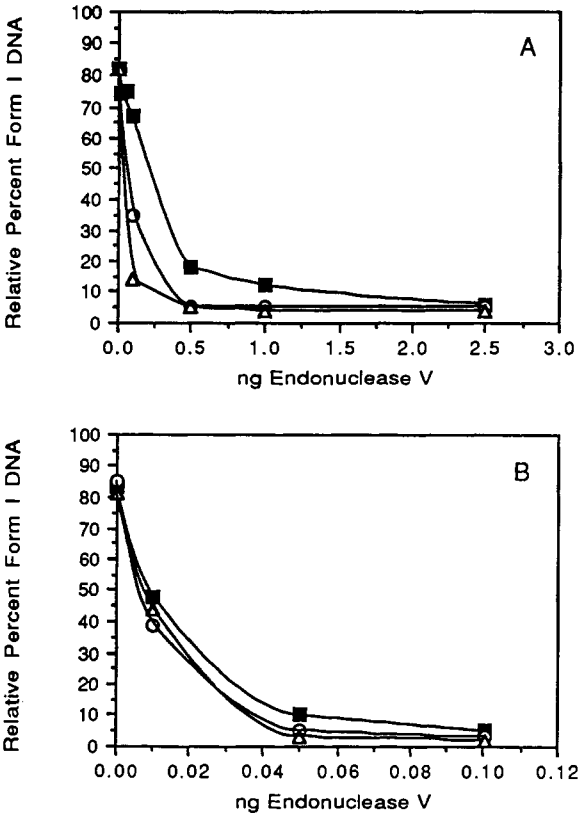

FIGURE 5: Analysis of T4 endonuclease V nicking of form I DNA containing apurinic sites. Partially purified endonuclease V was added to 1.0 μg of acid-depurinated ^{3}H-pBR322 in 20 μL of either (A) 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, and 1 mg/mL BSA or (B) 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C for 30 min. The three topological forms of DNA were separated on 1% agarose gel, and the relative mass fraction was determined by scintillation spectroscopy. All experiments were performed in duplicate. Endonuclease V (wild type) (■); Leu-60 (○); Leu-59, Leu-60 (△).

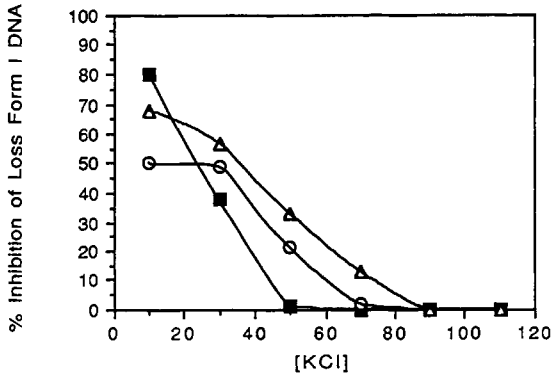

FIGURE 6: Inhibition of pyrimidine dimer specific nicking activity in the presence of unirradiated calf thymus DNA. Partially purified endonuclease V was added to 1.0 μg of UV-irradiated ^{3}H-pBR322 in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, various concentrations of KCl, and 1 mg/mL BSA either in the presence or in the absence of purified sonicated calf thymus DNA. The fraction of form I DNA remaining after a 30-min incubation at 37 °C in the presence or absence of a 10-fold excess of unirradiated competitor DNA was determined at KCl concentrations of 10, 30, 50, 70, 90, and 110 mM KCl. Percent inhibition of form I DNA loss in the presence of competitor calf thymus DNA was determined by a comparison to control reactions containing no calf thymus DNA. Endonuclease V (wild type), 1 ng/assay (■); Leu-60, 1 ng/assay (○); Leu-59, Leu-60, 1 ng/assay (△).

monomer–dimer equilibrium in solution. Our results and those of Minton et al. (1975) demonstrate that under low-salt conditions the monomer state is highly favored. Under high-salt conditions, we observe an equilibrium shift, favoring dimer formation with increasing endonuclease V concentration. Both hydrophobic and Coulombic forces are expected to contribute to the formation of a stable wild-type endonuclease V dimer. It is also probable that endonuclease V has highly basic surface characteristics. Thus, in low ionic strength solutions, the hydrophobic and Coulombic forces which should promote protein–protein association are weak relative to the repulsive force of the high charge distribution. By contrast, in a high ionic strength environment, the contribution of the charged character which may interfere with endonuclease V–endonuclease V interaction is masked by counterions in the solution. This masking allows the attractive forces to promote the resulting equilibrium shift toward a dimeric state.

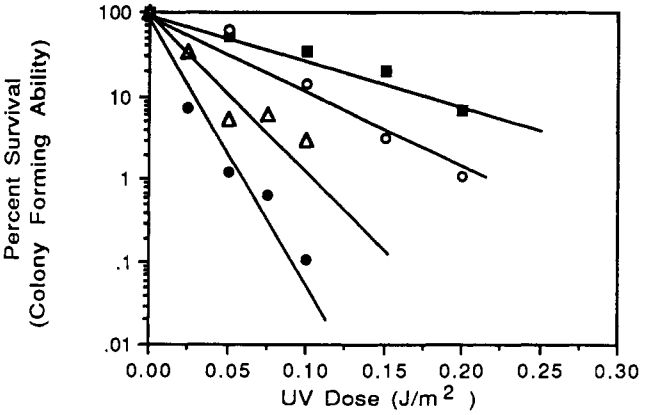

FIGURE 7: Colony-forming ability of UV-irradiated repair-deficient *E. coli* cells containing various *den*V gene constructs. AB2480 with pGX2608-*den*V$^-$ (●); pGX2608-*den*V$^+$ (■); pGX2608-*den*V-Leu-60 (○); pGX2608-*den*V-Leu-59, Leu-60 (△).

An apparent perturbation in this high-salt monomer–dimer equilibrium was observed for mutant endonuclease V molecules containing conservative mutations in the putative dimer interaction site. Although the hydrophobic nature of the mutation sites was maintained during the Phe-60 → Leu-60 and Phe-59, Phe-60 → Leu-59, Leu-60 alterations, these subtle changes have destabilized potential dimer formation. Thus, in a high ionic strength solution, the hydrophobic component of stable dimer formation has been weakened, and the remaining attractive Coulombic forces are unable to maintain the dimer state. The result is an equilibrium shift toward the monomer state despite the ionic strength of the solution.

The decreased protein dimerization capacity of the Leu-60 and Leu-59, Leu-60 mutant enzymes had an effect on the mechanism by which endonuclease V incises at pyrimidine dimers and AP sites. Under low-salt conditions when both the wild-type and mutant proteins exist as solution monomers of endonuclease V, the mechanism by which the enzyme mutants locate pyrimidine dimers has become less processive. Similarly, although not experimentally demonstrated, one can envision a like scenario for the incision of AP-containing DNA. At low-salt levels, mutant AP incision occurred as a result of the random interaction of a more distributive search. This yielded an increased rate of form I plasmid DNA loss relative to the processive incision of AP sites by the wild-type enzyme. Under high-salt conditions, in contrast to wild-type endonuclease V, the Leu-60 and Leu-59, Leu-60 mutants in solution remained as a monomeric population. This resulted in mutant enzymes with both a less highly processive scanning mechanism and a reduced pyrimidine dimer specific nicking activity. At high-salt levels, there was an increase in the rate of AP incision for both the mutant and wild-type enzymes resulting from a general reduction in DNA affinity. However, the relative

increase in the wild-type AP incision rate, resulting from the shift to a distributive search mechanism, was twice that of the mutant enzymes. Interestingly, the specific activity of the mutants was comparable to that of the wild-type enzyme under these conditions.

In other results from this laboratory (Augustine et al., 1991), the substitution of isoleucine for histidine at amino acid position 56 resulted in the creation of a mutant enzyme with pyrimidine dimer specific nicking characteristics similar to those described above. These characteristics typically indicate a reduced nontarget DNA affinity resulting in a distributive target search mechanism. However, when the pyrimidine dimer specific nicking activity of the Ile-56, Leu-60 and Leu-59, Leu-60 mutants was measured in the presence of competitor DNA, pyrimidine dimer specific nicking by the mutant enzymes was inhibited by competitive binding to unirradiated DNA. This competition was observed even at salt concentrations greater than the salt concentrations required for wild-type endonuclease V to exhibit a significantly reduced nontarget DNA affinity. We interpret these data to indicate that these mutants exhibit an increased affinity for nontarget DNA, despite the kinetic analyses that describe mutant proteins which have undergone a shift toward a more three-dimensional, low nontarget DNA affinity, target search. We believe that the resolution of this apparent discrepancy requires expanded considerations of factors other than nontarget DNA affinity which are involved in target location. In the case of endonuclease V, it appears that the oligomeric state of the enzyme is critical. This was revealed by the creation of mutants with both a reduced dimerization capacity and an increased nontarget DNA affinity in which the target search occurs via a less processive mechanism. It is also possible that the maintenance of the monomeric state exposes additional nonspecific DNA binding sites on the endonuclease V surface which act to increase the nontarget DNA affinity.

The intermediate levels of enhanced UV survival conferred by the expression of the Leu-60 and Leu-59, Leu-60 mutants in vivo are subject to several interpretations. The mutant enzymes accumulated at reduced levels within *E. coli* relative to the accumulation of wild-type enzyme. Perhaps this might reflect inefficient protein folding or the attainment of an unstable final structure (Vershon et al., 1986). If so, the reduced UV survival would be the result of limited cellular accumulation, and the altered solution and substrate interactions observed would be interpreted to be in vitro artifacts of an unstable protein. However, when the intracellular accumulation of the mutant enzymes is compared to that of wild-type endonuclease V, it is apparent that both the mutant and native enzymes accumulated at concentrations which are not limiting for cell survival (Recinos et al., 1986). Specifically, when repair-deficient *E. coli* containing the wild-type gene were grown under optimal conditions, endonuclease V accumulated to a level of 0.2–0.4% of the total cellular protein. Yet, when the same cells containing the wild-type gene were grown in 0.4% glucose, endonuclease V accumulated to approximately 0.01–0.02% of the total cellular protein. This reduced level of protein accumulation was shown to be slightly below saturating levels for maximal UV survival enhancement (Recinos et al., 1986). In this study, the potential dimer interaction mutant enzymes were present intracellularly under optimal conditions at approximately 10% of the wild-type level. Thus, the mutant enzymes accumulated to 0.02–0.04% of the total cellular protein. Since this level of accumulation is sufficient to meet the necessary protein concentration at which cell survival is no longer limited, the UV survival of cells expressing the mutant enzymes should be independent of the reduced amount of intracellular mutant enzyme.

Previous in vivo studies have shown that, although the *E. coli* intracellular cation concentration is considerably greater than 40 mM, endonuclease V acts processively under physiological conditions (Gruskin & Lloyd, 1988). The biological necessity of this processive action has also been demonstrated (Dowd & Lloyd, 1989a,b, 1990). With this in mind, we postulate that the oligomeric characteristics of endonuclease V in solution do not dictate in simple terms the manner in which the enzyme interacts with DNA in vitro and in vivo. Rather, it appears that at least a partial contributor to the processive diffusional mode is the stable formation of an endonuclease V dimer. We speculate that in vitro, at low-salt concentrations the repulsive force of a wild-type endonuclease V–endonuclease V interaction may be neutralized as the positive charges on the enzyme surface interact with the negatively charged nontarget DNA backbone. Thus, on the surface of the DNA, even at low-salt concentrations, a stable dimer could be formed which acts via a processive scanning mechanism. In vitro, at high-salt concentrations, nontarget DNA is not necessary to promote stable dimer formation. The dimer interacts directly, in a distributive manner, with nontarget DNA. When the ability of the enzyme to form a stable dimer is impaired by mutation, the enzyme acts less processively under low-salt conditions. Even though the enzyme monomers have a higher affinity for nontarget DNA, since protein–protein associations are disrupted, effective scanning cannot occur, and, thus, one observes destabilization of the nonspecifically bound state of the enzyme and increased dissociation from the DNA molecule prior to efficient target site location. As a consequence, the enzyme locates pyrimidine dimers in vitro via a limited processive mechanism. At high-salt concentrations, the distributive mechanistic mode is maintained. The reduced specific activity of the mutant enzymes could result from the reduced concentration of enzyme molecules present in the functional dimeric state or from the instability of the dimer state. Finally, the effect in vivo is a limited processive excursion as illustrated by the reduced colony-forming ability of the mutants relative to wild-type endonuclease V. Previous studies (E. Gruskin, unpublished observations) indicate that this in vivo extrapolation based on in vitro KCl concentration is sound. Leirmo et al. (1987) have demonstrated that the substitution of glutamate for chloride dramatically enhances the interaction of DNA with RNA polymerase and a variety of restriction endonucleases. Although the use of potassium glutamate to modulate ionic strength conditions may be more physiologically relevant, the unpublished studies mentioned above demonstrate that in vitro the ionic modulation of endonuclease V target search mechanisms with KCl is identical with the modulation observed with potassium glutamate.

We believe this work indicates that a dimeric interface occurs between like enonuclease V molecules along a putative α-helix containing amino acid residues His-56, Phe-59, and Phe-60. We also believe that the dimeric state of endonuclease V is necessary for DNA scanning. Such speculation is not unprecedented. *E. coli* lac repressor, *Bam*HI endonuclease, *Eco*RI endonuclease, and the bacteriophage λ cro protein all function as dimers and locate their target sequences by utilizing a processive one-dimensional diffusion mechanism. Perhaps coincidentally, Mossing and Sauer (1990) have also identified a key phenylalanine residue necessary for λ cro protein dimer formation. Only the co-crystallization of an endonuclease V dimer on DNA or the dimeric crystallization of endonuclease

V will unequivocally substantiate our speculation. Undoubtedly, DNA scanning is much more complex than can be described purely by electrostatic interactions.

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Quadruplex DNA Formation in a Region of the tRNA Gene supF Associated with Hydrogen Peroxide Mediated Mutations†

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ABSTRACT: A hot spot for H_2O_2/Fe-mediated mutation has been observed between bases 154 and 170 of the supF gene in the mutation reporter plasmid pZ189 [Moraes et al. (1990) *Carcinogenesis 11*, 283; Akman et al. (1991) *Mutat. Res.* (in press)]. To further characterize this hot spot, we synthesized the 33mer d(pAAAGTGATGGTGGTGGGGGAAGGATTCGAACCT) (pZ33), which is complementary to bases 159–191 of the supF gene. pZ33 annealed spontaneously in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl at 50 °C into two major forms, one of which migrates more slowly than does $d(pT)_{33}$ on nondenaturing 12% polyacrylamide gels. We propose that this form is a four-stranded structure stabilized by Hoogsteen-type deoxyguanosine quartets involving all deoxyguanosines of the sequence d(pGGTGGTGGGGG) because of the following. (1) pZ33 migrates as a single form that comigrates with $d(pT)_{33}$ on denaturing 20% acrylamide–8 M urea gels. (2) Annealing an equimolar mixture of 5′-^{32}P-labeled pZ33 and the oligodeoxynucleotide d(pTTTTTTTTpZ33TTTTTTTT) (pZ49), as well as 5′-^{32}P-labeled pZ49 and pZ33, caused the formation of four, discreet slowly migrating bands on nondenaturing 12% polyacrylamide gels. Mixing 5′-^{32}P-labeled pZ33 with 5′-^{32}P-labeled pZ49 resulted in five slowly migrating bands. (3) An oligodeoxynucleotide identical with pZ33 except that every deoxyguanosine has been replaced with deoxyinosine did not anneal into a slowly migrating form. (4) Dimethyl sulfate protection studies demonstrated that all deoxyguanosines of the sequence d(pGGTGGTGGGGG) were protected at N-7 in the slowly migrating form but not in single-stranded pZ33. These data suggest that a hot spot for H_2O_2/Fe-mediated base substitutions is located adjacent to a sequence that can spontaneously adopt a quadruplex structure in which deoxyguanosine quartets are Hoogsteen bonded.

Four-stranded complexes composed of sets of guanosine residues Hoogsteen bonded in deoxyguanosine quartets were first proposed by Gellert et al. (1962) on the basis of X-ray diffraction patterns obtained from fibers of polyguanylic acid [poly(G)][1] and gels formed by guanylic acid. These structures are remarkably stable. For poly(G) the alkali-induced helix–coil transition does not occur until pH 11.2 at 25 °C, and the thermally induced transition is not complete even at 100 °C at neutral pH (Fresco & Massoulie, 1963). This exceptional stability and the capacity of the mononucleotide to form an extended complex may be due in part to the unusual stacking energy of the guanosine quartet (Arnott et al., 1974) and in part to the capacity of O-6 of guanine to participate in a coordination complex involving either Na^+ or K^+, as subsequently suggested for the stabilization of telomeric DNA (Sundquist & Klug, 1989; Williamson et al., 1989; Sen & Gilbert, 1990). The RNA structures appear to form with all four strands in a parallel orientation (Arnott et al., 1974; Zimmerman et al., 1975).

More recently, structures containing deoxyguanosine quartets have been suggested for oligodeoxynucleotides corresponding to the immunoglobulin switch region (Sen & Gilbert, 1988), the region containing codon 12 of the human c-Ha-*ras* gene (Smith et al., 1989), and the telomeric repeat sequences of Tetrahymena (Sundquist & Klug, 1989) and *Oxytrichia* (Williamson et al., 1989). While the oligodeoxynucleotides from the immunoglobulin switch region and c-Ha-*ras* appear to form parallel quadruplexes similar to the structure proposed for poly(G), the telomeric sequences form antiparallel structures composed of sets of foldback molecules.

In light of the occurrence of these structures at sites of spontaneous gene rearrangement (Sen & Gilbert, 1988) and point mutation (Smith et al., 1989), it is important to ask whether a relationship exists between the spectrum of mutations induced by DNA-damaging agents and the potential for unusual structure formation in target DNAs. To approach this question, we prepared a map of mutations occurring in the supF gene replicating in the reporter plasmid pZ189 (Seidman et al., 1985) after exposure to hydrogen peroxide/Fe (Akman et al., 1991). This map is in agreement with that of Moraes et al. (1989) for hydrogen peroxide/Fe-mediated mutations resulting from the use of similar mutagenesis techniques. Both maps identify hot spots for mutation adjacent to sequences having the properties appropriate for the formation of unusual structures, e.g., triplexes (Beal & Dervan, 1991) or quadruplexes (Sen & Gilbert, 1990). In this report,

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[1] Abbreviations: TBE buffer, 89 mM Tris base–89 mM boric acid–2 mM EDTA, pH 8.3; poly(G), polyguanylic acid.

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