Oligonucleotide Site Directed Mutagenesis of All Histidine Residues within the T4 Endonuclease V Gene: Role in Enzyme–Nontarget DNA Binding†

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ABSTRACT: In order to evaluate the contributions that histidine residues might play both in the catalytic activities of endonuclease V and in binding to nontarget DNA, the technique of oligonucleotide site directed mutagenesis was used to create mutations at each of the four histidine residues in the endonuclease V gene. Although none of the histidines were shown to be absolutely required for the pyrimidine dimer specific DNA glycosylase activity or the apurinic lyase activity, conservative amino acid changes at His16 produced enzymes with little or no catalytic activity. In addition, the evaluation of conservative and radical amino acid substitutions at positions 34, 56, and 107 is consistent with the interpretation that each of these histidines may be involved in nontarget DNA binding. The data supporting this conclusion are that histidine changes to lysine at positions 34 and 107 enhance the nontarget DNA binding activity of the mutant enzymes while neutralization of charge at His56 reduces nontarget DNA binding.

T4 endonuclease V is a pyrimidine dimer specific DNA repair initiating enzyme which locates its substrate through a one-dimensional search mechanism (Lloyd et al., 1980; Gruskin & Lloyd, 1986; Ganesan et al., 1986; Dowd & Lloyd, 1989a,b, 1990). As the enzyme slides on nontarget DNA and encounters a pyrimidine dimer, it binds to the dimer and sequentially catalyzes glycosylic bond scission on the 5′-pyrimidine of the dimer and, by way of β-elimination, cleaves the phosphodiester bond between the two pyrimidines of the dimer (Gordon & Haseltine, 1980; Radany & Friedberg, 1980; Seawell et al., 1980; McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981; Nakabeppu et al., 1982; Manoharan et al., 1988; Mazumder et al., 1989). For a review, see Dodson and Lloyd (1989). Endonuclease V is only one example of a growing list of specific DNA glycosylases which also contain an associated apurinic/apyrimidinic (AP)[1] lyase activity: *Micrococcus luteus* UV endonuclease (Gordon & Haseltine, 1980; Haseltine et al., 1980; Hamilton & Lloyd, 1989), endonuclease III (Bailly & Verly, 1987; Kow & Wallace, 1987; Asahara et al., 1989; Cunningham et al., 1989), and (formamido)pyrimidine DNA-glycosylase (O'Connor & Laval, 1989; Boiteaux et al., 1990).

In order to gain insight into the chemical mechanism by which endonuclease V catalyzes the breakage of the phosphodiester bond at an AP site, Kim and Linn (1988) sequentially treated AP DNA with endonuclease V and human fibroblast AP endonuclease. The resulting carbohydrate product was analyzed and chromatographically shown to be consistent with a β-elimination product and not deoxyribose 5-phosphate. The mechanism of incision was established by β-elimination of the 3′-phosphate (Manoharan et al., 1988). They utilized ^{13}C NMR spectroscopy to characterize the terminal carbohydrate as an α,β-unsaturated aldehyde. In further investigations, Mazumder et al. (1989) have used stereospecifically labeled abasic sites to analyze the stereospecificity of hydrogen abstraction in the β-elimination reaction. Using ^{1}H NMR spectroscopic analyses, they were able to show that endonuclease V abstracts the *pro-S* 2-hydrogen and that the stereochemistry of the reaction is syn. The final product was an α,β-unsaturated aldehyde with trans geometry. Although the details of these chemical reactions are being established, there is no information on which amino acid residues within endonuclease V are responsible for carrying out these bond scissions. In order to assess the role that certain amino acids might play in enzyme catalysis, we have utilized the analyses of Zvelebil and Sternberg (1988), who characterized the structural features of catalytic residues in proteins of known structure and defined catalytic mechanism. Within these proteins, surprisingly 33% of all catalytic residues were His even though His only comprises 2.3% of the total amino acids. These authors rationalize that since the imidazole ring of His can be charged or neutral at physiological pH, it is very suitable for catalyzing chemical reactions. In contrast to His, Lys was only found as a catalytic residue 3% of the time while from amino acid composition data, Lys occurs with a frequency of 6.3%. Thus, we have chosen to investigate the role that histidine residues within endonuclease V might play in the catalytic mechanisms of the enzyme. Since there are only four His codons within the endonuclease V gene, we have used the technique of oligonucleotide site directed mutagenesis to change those sites.

In addition, the *M. luteus* UV endonuclease shares many of the same physical and enzymatic properties with endonuclease V. Both enzymes have been shown to scan nontarget DNA in vitro under similar conditions of ionic strength. The in vitro DNA scanning capacity of UV endonuclease can also be modulated by alterations in the pH of the nicking reaction (Hamilton & Lloyd, 1989). It has been proposed that histidine residues might be responsible for mediating this effect. As a result, we have investigated whether the histidines within

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[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; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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

strain, plasmid, or phage	genotype or phenotype	source
E. coli		
UT481	*met thy* Δ (*lac-pro*) *hsdRBamHi hsdM*$^+$ *supD* Tn*10*/F′ *traD36 proAB lacIqZ*Δ M15	C. Lark, University of Utah
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. Ganesan, Stanford University
plasmid		
pGX2608	Ap^{r}λ O_LP_R λ t_{4s} $galK_+$	Genex Corp.
pGX2608-16-*den*V$^+$	Ap^{r}λ O_LP_R endonuclease V$^+$ λ t_{4s} galK$^+$	this laboratory
pGX2608-16-*den*V His16 → Arg16	H016R endonuclease V	this study
pGX2608-16-*den*V His16 → Lys16	H016K endonuclease V	this study
pGX2608-16-*den*V His16 → Thr16	H016T endonuclease V	this study
pGX2608-16-*den*V His16 → Tyr16	H016Y endonuclease V	this study
pGX2608-16-*den*V His34 → Lys34	H034K endonuclease V	this study
pGX2608-16-*den*V His56 → Ile56	H056I endonuclease V	this study
pGX2608-16-*den*V His107 → Lys107	H107K endonuclease V	this study
phage		
M13mp18-O_LP_R *den*V		Recinos & Lloyd (1986)
M13mp18-O_LP_R *den*V His16 → Arg16		this study
M13mp18-O_LP_R *den*V His16 → Lys16		this study
M12mp18-O_LP_R *den*V His16 → Thr16		this study
M13mp18-O_LP_R *den*V His16 → Tyr16		this study
M13mp18-O_LP_R *den*V His34 → Lys34		this study
M13mp18-O_LP_R *den*V His56 → Ile56		this study
M13mp18-O_LP_R *den*V His107 → Lys107		this study

the native endonuclease V contribute to nontarget DNA binding and scanning.

Materials and Methods

Bacteria, Phage, and Plasmids. The *Escherichia coli* strains that were used throughout this study are described in Table I. Plasmids and M13 phage constructs carrying *den*V gene mutants are also described in Table I.

Oligonucleotide Site Directed Mutagenesis of the denV Gene. The construction of mutants within the *den*V gene was carried out by using M13 mp18-O_LP_R*den*V single-stranded circular DNA as a template. The construction of this bacteriophage and a detailed description of the site-directed mutagenesis procedures have been previously published (Recinos & Lloyd, 1988). Synthetic oligonucleotides which were used to create the desired changes within the gene were synthesized on a Biosearch Cyclone DNA synthesizer and were purified as previously described (Lloyd et al., 1986). Since the *den*V gene was subcloned such that the coding strand is packaged in the (+) strand of the M13 phage, the following noncoding strand, single-stranded oligonucleotides were synthesized and purified to create each of the following mutants:

(1) H016R ^{5}′AGCCATTAAACGTTGGTCAGC^3′;

(2) H016K, ^{5}′CAGCCATTAATTTTTGGTCAGCC^3′;

(3) H016A, ^{5}′AGCCATTAAAGCTTGGTCAGC^3′;

(4) H016I, ^{5}′AGCCATTAAGATCTGGTCAGC^3′;

(5) H016Y, ^{5}′AGCCATTAAGTATTGGTCAGC^3′;

(6) H016T, ^{5}′AGCCATTAAAGTTTGGTCAGC^3′;

(7) H034K, ^{5}′CGTTAGCAACTTTCTTACGAACT^3′;

(8) H056K, ^{5}′AGAATGTAACTTTACCTGCGCCA^3′;

(9) H056I, ^{5}′GAATGTAACGGATCCTGCGCC^3′;

(10) H056R, ^{5}′GAATGTAACACGACCTGCGCC^3′;

(11) H056A, ^{5}′GAATGTAACGGCGCCTGCGCC^3′;

(12) H107K, ^{5}′TAGAAGCTTCTTTGGGAATATAA^3′.

The bases which are underlined are those which differ from the wild-type sequence.

Protein Purification of Mutants of Endonuclease V. Following the successful site-directed mutagenesis of the *den*V gene at various sites (as judged by complete DNA sequence analysis in the mutated region), the altered gene was subcloned into an expression plasmid, pGX2608. The detailed procedures for subcloning and verifying the orientation of the DNA insert have been previously published (Recinos & Lloyd, 1986; Recinos et al., 1986). The *den*V gene was expressed from a hybrid λ promoter, O_LP_R, which is composed of the leftward operator of phage λ fused to the rightward promoter of phage λ. The origin of DNA replication is protected by the λ4S terminator (*oop* terminator). Plasmids which contained the correct orientation of the *den*V gene were transformed into *E. coli* AB2480 which are both excision repair deficient (*uvr*A$^-$) and recombination repair deficient (*rec*A$^-$). For each mutant that was analyzed, 1-L cultures were grown at 30 °C for 16 h. The cells were pelleted by centrifugation (5000*g*) and resuspended in 100 mL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 10% (v/v) ethylene glycol (buffer A) at 4 °C. The cells were broken by sonication and debris removed by centrifugation (10000*g*) for 20 min. The supernatant was loaded onto a 200-mL bed volume single-stranded DNA–agarose column, and after an extensive washing of the matrix with buffer A (500 mL), a linear salt gradient was run from buffer A to buffer A supplemented with 2 M KCl. All mutant proteins bound to the single-stranded DNA–agarose column at low salt concentrations and eluted at ~700 mM KCl. The samples were desalted by the passage of active fractions over a Sephadex G100 column in 25 mM NaH_2PO_4 (pH 6.8), 1 mM EDTA, 100 mM KCl, and 10% (v/v) ethylene glycol. In some cases, it was necessary to concentrate the mutant endonuclease V using heparin–agarose chromatography. The mutant enzymes bind to the heparin–agarose column at low salt and elute at ~300 mM KCl. The amount of endonuclease V was quantitated by Western blot analyses using mouse polyclonal antibodies which had been raised against wild-type endonuclease V. Detailed quantitation methods have been previously published (Dowd & Lloyd, 1989b). Briefly, active fractions from the heparin column were combined, and various amounts of those samples and pure

endonuclease V were run on an SDS–15% polyacrylamide gel. The proteins were transferred to nitrocellulose paper in 5 mM Tris-HCl (pH 8.0), 30 mM glycine, and 20% methanol at 290 mA for 16 h. The nitrocellulose paper was blocked in 3% gelatin in 50 mM Tris-HCl (pH 8.0) and 1.25 M NaCl for 1 h at 37 °C. The blocking solution was discarded and primary antibody added and incubated for 20 h at 37 °C. The nitrocellulose sheet was washed 3 times in 50 mM Tris-HCl (pH 8.0) for 5 min each, and a 1:1000 dilution of affinity-purified rabbit anti-mouse IgG horseradish peroxidase conjugate was added. After the secondary antibody reacted for 3 h at 37 °C, the nitrocellulose was washed as described above. The immunoreactive bands were developed as follows: 32 mg of 4-chloro-1-naphthol in 12 mL of methanol was added to 60 mL of 50 mM Tris-HCl (pH 8.0), 1.25 M NaCl, and 120 μL of 30% H_2O_2. Following color development, the nitrocellulose was rinsed in distilled H_2O, dried, and scanned. The peak areas of the endonuclease V proteins were compared to a standard of pure endonuclease V, and the concentration of enzyme was directly extrapolated from the standard curve.

Cell Survival following Ultraviolet Irradiation. A detailed procedure for measuring the colony-forming ability of wild-type and mutant *den*V gene constructs has been previously published (Dowd & Lloyd, 1989b).

Pyrimidine Dimer Specific Nicking Assay. ^{3}H-pBR322 (~4000 cpm/μg) in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (1 μg/assay point) was irradiated for 4 min, 5 s at a fluence of 100 μW/cm^2 in order to generate 25 pyrimidine dimers per molecule. This DNA was diluted into either a "low" or a "high" salt reaction buffer: 10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, and 10% (v/v) ethylene glycol or 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, and 10% (v/v) ethylene glycol, respectively. Various concentrations of partially purified endonuclease V were added to the reaction buffer and the reactions incubated at 37 °C. Aliquots were removed at 15 and 30 min and the reactions terminated by the addition of SDS to a final concentration of 0.75%. The three forms of DNA were separated by agarose gel electrophoresis and visualized by staining with 0.5 μg/mL ethidium bromide in electrophoresis buffer. Form I, II, and III DNAs were excised from the gel under long-wavelength UV light (280 nm) and placed in scintillation vials with 100 μL of 1 N HCl. The vials were briefly autoclaved to melt the agarose, and then 10 mL of aqueous scintillation cocktail was added to each vial. The percentage of each form of DNA was determined from the total radioactivity in each lane.

For experiments in which the pyrimidine dimer specific DNA glycosylase activity was measured in addition to the complete incision reaction, the same procedure as described above was followed except that after the reactions had been terminated by the addition of SDS, the final pH of the DNA-containing solution was raised to 11–11.5 by the addition of NaOH. Samples were incubated for 30 min at 37 °C in order to hydrolyze alkaline-labile sites and then neutralized by the addition of HCl. DNA samples were further processed as previously described.

For dimer-specific DNA nicking assays in which the effects of $AgNO_3$ were tested, the final concentration of $AgNO_3$ was 2.5 mM.

For pyrimidine dimer specific nicking assays in which the size distributions of single-stranded DNA fragments were analyzed, the time course nicking reactions at pH 8.0 were performed as described above except that after the reaction was terminated NaOH was added to a final concentration of 100 mM. The DNAs were loaded onto an alkaline agarose gel which had been prepared as follows: a 1% (w/v) agarose gel in 40 mM sodium acetate and 1 mM EDTA was prepared, and after solidification, the gel was removed from the electrophoresis apparatus and soaked for 16 h with two changes of 30 mM NaOH. DNA nicking reactions at pH 6.0 were performed in the following buffer: 50 mM MES (pH 6.0) and 100 mM NaCl. The single-stranded DNA fragments and collapsed unreacted form I DNAs were separated according to size through the agarose gel, transferred to nitrocellulose paper, and probed with ^{32}P nick-translated pBR322 (~10^7 cpm/μg). Kodak XAR5 film was used for the autoradiograms. The choice of the individual lanes which were shown as representatives for pH 6.0 versus pH 8.0 was based on an ~30% form I DNA remaining.

Apurinic Nicking Assay. ^{3}H-Apurinic DNA was prepared as follows: 40 μg of form I ^{3}H-pBR322 DNA was incubated for 10 min at 63 °C in 10 mM sodium citrate (pH 4.0) and 100 mM NaCl, followed by an addition of an equal volume of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The DNA was immediately precipitated with 2.5 volumes of 95% ethanol. After 1 h in an ethanol–dry ice bath, the DNA was pelleted, dried, and resuspended in water. Apurinic nicking assays were performed in 5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 50 mM KCl, and 5% (v/v) ethylene glycol and various concentrations of wild-type and mutant enzymes. Time points were removed at 1, 2.5, 5, and 15 min and the reactions terminated by the addition of SDS to 1%.

Assessment of Wild-Type and Mutant Enzyme Affinity for Nontarget DNA-A Pyrimidine Dimer Specific Nicking Assay in the Presence of Competitor Unirradiated DNA. A series of kinetic pyrimidine dimer specific nicking assays were performed at 10, 30, 50, 70, 90, and 110 mM KCl in the presence and absence of a 10-fold excess of unirradiated competitor DNA. The basic reaction mixture per time point contained the following: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, *X* mM KCl, and 1 μg of ^{3}H-pBR322 DNA which contained 25 pyrimidine dimers per molecule, ±10 μg of purified, sheared calf thymus DNA where *X* = 10, 30, 50, 70, 90, or 110 mM KCl. The amount of wild-type enzyme and each mutant which was used per time point was as follows: wild type, 1.4 ng; H034K, 2 ng; H056I, 1.5 ng; H107K, 3.0 ng. The time points which were used in these analyses were as follows: wild type, 5 and 10 min; H034K, 10 and 20 min; H056I, 10 and 20 min; H107K, 10 and 20 min. The reactions were terminated and processed as previously described.

Results

Introduction of Mutations at Histidine Residues within the Endonuclease V Gene, denV. Previously, the *den*V structural gene had been subcloned into M13 mp18 RF DNA. The single-stranded DNA from this phage was used as a template to engineer base changes in the *den*V gene such that His16 became Arg16 (H016R), His34 became Lys34 (H034K), His107 became Lys107 (H107K), and His56 became Ile56 (H056I) (Table I). The alteration of H056I was not the first choice of mutations to create at this site. Three separate attempts were made to create the H056K and H056R enzymes, but none of these attempts was successful even though other mutations were made on the same DNA template. However, no difficulty was encountered in making the H056I mutation. All alterations in the gene were verified by DNA sequence analysis. The mutated *den*V structural genes were subcloned with the correct orientation into the expression vector pGX2608 at a unique *Cla*I restriction enzyme site which is located immediately downstream of a hybrid phage λ promoter, $\lambda O_L P_R$. These plasmids were introduced into an ex-

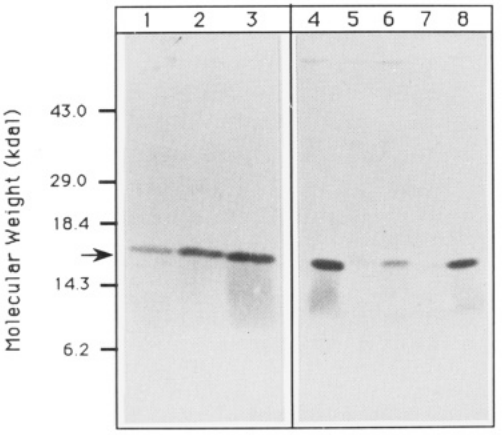

FIGURE 1: Western blot analysis of the endonuclease V proteins which accumulated within stationary-phase *E. coli* AB2480 grown at 30 °C. Lanes 1–3 contain 100, 200, and 400 ng of purified wild-type endonuclease V. Lanes 4–8 contain 50 μg of total cellular protein from cells producing wild-type endonuclease V (lane 4), H016R (lane 5), H034K (lane 6), H056I (lane 7), and H107K (lane 8). Polyclonal mouse anti-endonuclease V antibodies were used as the primary antibody.

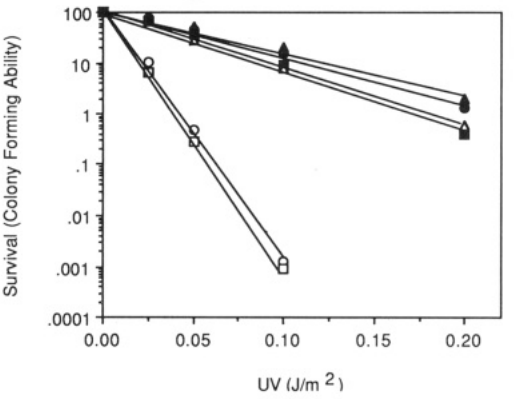

FIGURE 2: Colony-forming ability of UV-irradiated DNA repair deficient *E. coli* containing *den*V$^+$, *den*V$^-$, and mutated *den*V plasmids. *E. coli* AB2480 containing various plasmids were grown in LB supplemented with 100 μg/mL ampicillin for 16 h at 30 °C, and multiple dilutions of each culture were plated onto hard agar plates. Cells were irradiated with the indicated dose of UV light and incubated in the dark for ~30 h, and colonies were counted. Each data point represents the average of six independent determinations: (○) pGX2608 (*den*V$^-$); (●) pGX2608-16 (*den*V$^+$ wild type); (□) H016R; (■) H034K; (△) H056I; (▲) H107K.

cision repair deficient (uvrA$^-$) and a recombination-deficient (recA$^-$) *E. coli*, AB2480. Each of the enzymes was purified by sequential passage over a single-stranded DNA–agarose affinity column, Sephadex-G100 gel filtration column, and heparin–agarose affinity chromatography. The concentrations of the purified enzymes were determined by quantitative Western blot analysis. These analyses utilized a mouse polyclonal antibody directed against wild-type endonuclease V as the primary antibody and a rabbit anti-mouse IgG conjugated to horseradish peroxidase as the secondary antibody. A series of wild-type endonuclease V standards (50–500 ng) were used to generate the standard curve from which the mutant enzyme concentrations were determined. Western blot analyses of whole cell extracts revealed that the H107K and H034K enzymes accumulated to ~50% and 20%, respectively, that of wild type while the intracellular accumulations of the H016R and H056I enzymes were only ~5% (Figure 1). Bowie and Sauer (1989) have concluded that a low intracellular accumulation of mutated enzyme molecules probably reflects that these residues are involved in the structural integrity of the protein and thus mutants do not fold as efficiently as the wild-type protein.

UV Survival of Cells Harboring Mutant denV Genes. The UV survival of *E. coli* AB2480 cells harboring the mutated *den*V plasmids was determined (Figure 2). The cells producing either H034K (■), H056I (△), or H107K (▲) endonuclease V were able to enhance the UV survival of AB2480 to levels equal to or slightly less than that of cells harboring a plasmid which produces wild-type endonuclease V (●). However, cells expressing the H016R endonuclease V (□) displayed a UV survival which was indistinguishable from that of cells containing only the expression vector with no *den*V gene (○). Since the accumulation of H016R protein was not drastically reduced relative to the H056I mutant, it appeared that the single point mutation at the His16 codon had inactivated the enzyme.

Pyrimidine Dimer Specific Nicking Activity. The dimer-specific nicking activity of each mutant was determined by using two enzyme assay conditions in which the wild-type enzyme has been shown to locate pyrimidine dimers either by a random three-dimensional search mechanism (100 mM monovalent salt) or by a one-dimensional sliding along the DNA (25 mM monovalent salt). For the high-salt experiments, supercoiled, covalently closed circular ^{3}H-pBR322 DNA (form I DNA) was UV-irradiated to introduce ~25 pyrimidine dimers per DNA molecule. Various concentrations of wild-type or mutant enzyme were added to this DNA, and aliquots were removed at 15 and 30 min. Reactions were terminated by the addition of SDS to a final concentration of 1%. The percentages of form I, II, and III DNAs were determined after each form of DNA had been separated by agarose gel electrophoresis. At the high salt concentration (open and closed circles), the activity of each of the altered enzymes except H016R was near that of the wild-type enzyme in that form I DNA was primarily converted into form II DNA. It is important that there was no significant accumulation of form III DNA until after most of the form I DNA has been incised, and only the addition of 5 and 10 ng of enzyme resulted in a significant accumulation of double-stranded breaks (Figure 3). Since double-strand breaks are produced by incising DNA at dimer sites in close proximity in complementary strands and are manifested when the majority of all pyrimidine dimer sites have been nicked, the lag in the production of form III DNA is characteristic of a distributive nicking reaction in which dimers are being randomly incised. An exception to this was H056I in which no major increase in form III DNA was observed. The remaining percentage of DNA at each point was form II DNA but was not shown in order to simplify the figures. The relative specific activity of each enzyme is given in Table II.

Table II: Relative Specific Activities of Endonuclease V Enzymes

enzyme	slope[a]	% rel sp act.[b]
wild type	1.87	100
H016R	0.05	3
H034K	1.50	80
H056I	0.95	51
H107K	1.87	100

[a] Slope of the (−ln form I DNA) − (−ln form I DNA$_{t=0}$)/ng of enzyme. [b] Percent relative specific activity = (slope of mutant enzyme/slope of wild-type enzyme)100.

By use of the same assay system as described above except that the final salt concentration was ~25 mM, the wild-type and mutant enzymes were tested for their ability to scan nontarget DNA by a one-dimensional diffusion mechanism. The experimental manifestation of such a mechanism is an

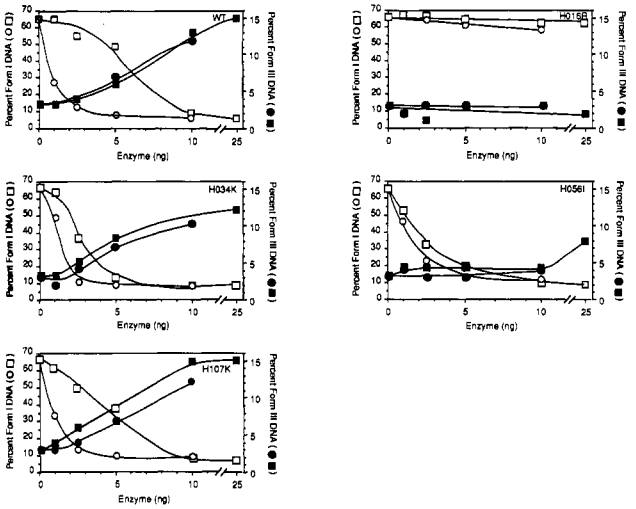

FIGURE 3: Pyrimidine dimer specific nicking assays at high and low salt concentrations. ^{3}H-pBR322 was UV-irradiated (25 pyrimidine dimers per DNA molecule) and used as a substrate for several concentrations of purified preparations of wild-type and mutant endonuclease V. The 100 mM salt concentration (open and closed circles) represents assay conditions in which the wild-type enzyme utilizes a random search mechanism for locating and incising DNA at the site of pyrimidine dimers. Aliquots were removed at 15 and 30 min. The data which are shown are as follows: for wild type, H016R and H034K, 30 min; for H056I and H107K, 15 min. Double-strand breaks (which yield form III DNA) arise from the incision of two dimers in close proximity on complementary DNA strands. The remainder of the DNA was form II (nicked circular) DNA, but those data were not shown in order to simplify the panels. The 25 mM salt concentration (open and closed squares) represents assay conditions in which the wild-type enzyme utilizes a one-dimensional scanning mechanism for locating pyrimidine dimer sites in plasmid DNA. The experimental design was the same as described above.

immediate and linear accumulation of form III DNA while there is still a significant fraction of DNA molecules with no incisions (form I DNA). Figure 3 (open and closed squares) shows that the wild-type H034K and H107K enzymes displayed nearly identical mechanisms of target site location by virtue of a linear increase in form III DNA while there were large amounts of unreacted form I DNA remaining. H056I demonstrated a significant lag in form III DNA production while H016R showed no dimer-specific nicking activity at this salt concentration. It should be noted that although the rate at which all dimers are incised is roughly equal between the analyses at high and low salt, the mechanism of target site location is different.

Pyrimidine Dimer Specific DNA Glycosylase Activity of H016R. Since the assays which were described in the previous section relied on the complete two-step breakage activities in order to detect a catalytic event, the DNA glycosylase activity of the H016R mutant was tested. If the H016R enzyme could make the glycosylic bond scission and not the apurinic incision, then after a nicking reaction is terminated, alkali treatment of an aliquot of the reacted DNA would result in single-strand breaks and a concomitant loss in form I DNA. However, the results of these experiments demonstrated that no glycosylic bond scissions were introduced into this DNA by the H016R endonuclease V (data not shown).

Binding of Mutant and Wild-Type Endonuclease V to Unirradiated DNA. Previously it has been demonstrated that endonuclease V binds to unirradiated DNA through electrostatic interactions between several basic residues on the enzyme and the acidic sugar–phosphate backbone of DNA (Lloyd et al., 1980; Gruskin & Lloyd, 1986; Ganesan et al., 1986; Dowd & Lloyd, 1989a,b, 1990; Nickell & Lloyd, 1991). Thus, at

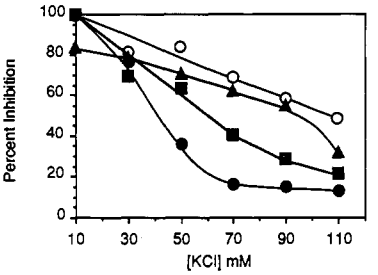

FIGURE 4: Inhibition of pyrimidine dimer specific nicking activity with unirradiated calf thymus DNA. Purified preparations of wild-type and catalytically active mutants of endonuclease V were added to 1 μg of ^{3}H-pBR322 DNA in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, various concentrations of KCl (10, 30, 50, 70, 90, 110 mM), and ±10 μg of purified sonicated calf thymus DNA. The amounts of each enzyme used and the length of each reaction time are given under Materials and Methods. The percent inhibition was calculated as follows: $1 - [(\text{form } I_{t_0} - \text{form } I_t \text{ with competitor DNA})/(\text{form } I_{t_0} - \text{form } I_t \text{ without competitor DNA})]100$ where form I_{t_0} represents the starting percentage of form I and form I_t represents the percentage of form I after a specified reaction time. (●) WT; (▲) H056I; (○) H034K; (■) H107K.

monovalent salt concentrations <40 mM, the wild-type endonuclease V binds to unirradiated DNA. This binding is measured by monitoring the rate of incision of UV-irradiated supercoiled DNA in the presence of a 10-fold excess of unirradiated DNA at various salt concentrations (Gruskin & Lloyd, 1986; Nickell et al., 1991). At salt concentrations >40 mM, the endonuclease V loses its ability to bind unirradiated DNA, and thus the 10-fold excess of unirradiated DNA no longer serves as an inhibitor of the dimer-specific nicking reaction. The salt concentration at which the mutated enzyme loses its affinity for unirradiated DNA relative to wild type is a good measure of its relative nontarget DNA binding affinity. UV-Irradiated ^{3}H-pBR322 DNA was mixed with a 10-fold excess of unlabeled calf thymus DNA at various salt concentrations, and wild-type or catalytically active mutant endonuclease V was added to the DNAs. The results in Figure 4 demonstrate that the salt concentration required to achieve a 50% reduction in the rate of incision of UV-irradiated plasmid for each of the catalytically active altered enzymes was considerably greater than that required for the wild-type enzyme. In this regard, the effect of the H034K enzyme was extremely dramatic, showing a 50% inhibition at ~110 mM, a result which represents a 3-fold increase in the salt concentration required to inhibit nontarget DNA binding. The wild-type inhibition was at ~40 mM, a result which is consistent with previously published data (Gruskin & Lloyd, 1986; Nickell & Lloyd, 1991).

Lack of Evidence for a pH-Dependent Modulation of DNA Scanning Activity. Previously, Hamilton and Lloyd (1989) demonstrated that the nontarget DNA binding function of another pyrimidine dimer specific endonuclease, *Micrococcus luteus* UV endonuclease, could be dramatically altered by modulation in the pH at which the nicking reactions were performed. The nontarget DNA binding was manifested by a processive nicking activity on UV-irradiated DNA. The processivity of *M. luteus* UV endonuclease was considerably greater at pH 6.0 versus pH 8.0, a result which suggested that histidine residues might be responsible for modulating this difference.

In order to test whether endonuclease V can be similarly modulated by the pH of the reaction conditions, we first examined whether changes in pH could significantly alter the mechanism for target site location. Reactions using *M. luteus* UV endonuclease were performed as positive controls for re-

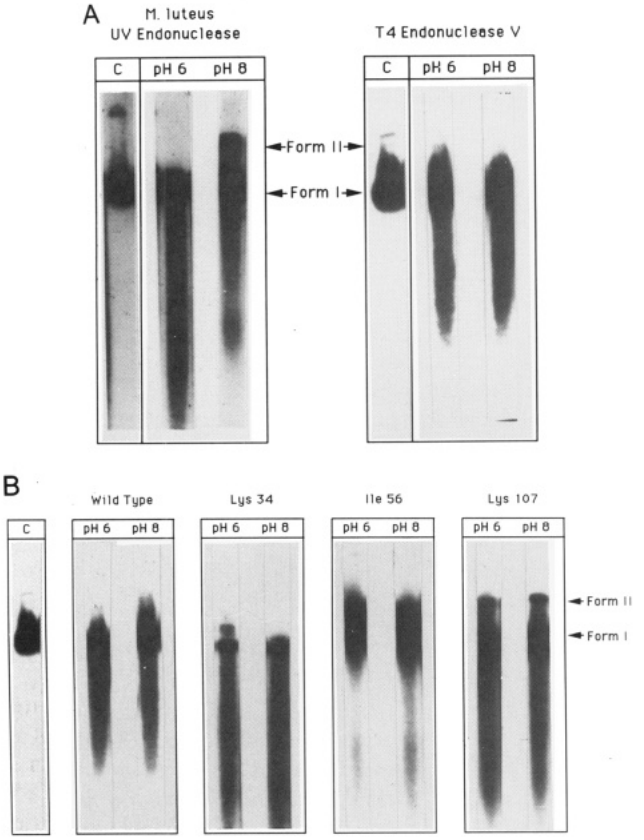

FIGURE 5: Effect of changes in the pH on the nicking reaction of *M. luteus* UV endonuclease, wild-type endonuclease V, and histidine mutant endonuclease V. For the data shown in panel A, pBR322 DNA was UV-irradiated to introduce 25 pyrimidine dimers per molecule, and the DNA was diluted into a pH 6.0 or 8.0 buffer (see Materials and Methods for details). UV endonuclease from *M. luteus* or endonuclease V was added to the DNA, and aliquots were taken at various times. Samples which were shown to contain ~30% form I DNA remaining were denatured, and the DNA was separated through alkaline denaturing gels. The DNAs were transferred to nitrocellulose paper and probed with ^{32}P-pBR322. Panel B shows a comparison of the wild-type endonuclease V at pH 6 and 8 with each of the histidine mutants, H034K, H056I, and H107K. The methodologies used were the same as described above.

actions which should show significant differences at pH 6 versus pH 8. UV-Irradiated ^{3}H-pBR322 was incubated with UV endonuclease or endonuclease V such that ~30% form I DNA was remaining. An aliquot of the DNA from each reaction was denatured by the addition of NaOH to 100 mM. The fragment size distribution of DNA provides a measure of the scanning and incision activities of the enzyme. Figure 5A shows a comparison of the pH-dependent modulation of scanning by the UV endonuclease (left panel) while the right panel shows data which demonstrate that endonuclease V does not show any apparent fragment size differences for reactions carried out at pH 6.0 versus pH 8.0.

Since the pH-dependent modulation of scanning was hypothesized to be mediated by histidine residues, the catalytically active, altered enzymes were analyzed at pH 6.0 and 8.0 (Figure 5B). As previously shown for the wild-type enzyme, none of the mutants displayed an altered accumulation of single-stranded breaks within their DNA as a function of the pH of the reaction. The H056I enzyme demonstrated a single-stranded DNA fragment size distribution which is consistent with a distributive or a very limited processive DNA scanning mechanism. The size distribution of single-stranded DNA fragments generated by the H034K and H107K enzymes appears to be slightly smaller than that observed for the wild-type enzyme. These results are in good agreement with the nontarget DNA binding study and strongly suggest that the H034K and H107K enzymes have an enhanced affinity for nontarget DNA.

Apurinic Nicking Activity. The second pyrimidine dimer specific catalytic reaction of endonuclease V is a phosphodiester backbone incision which can only occur after *N*-glycosyl bond scission. However, this phosphodiester bond scission can also occur on DNA containing abasic sites. Thus, in order to evaluate the involvement of histidines in the second catalytic activity, apurinic (AP) DNA was made by acid–heat treatment and used as a substrate. The relative AP nicking activity of the four histidine mutants was measured, and although H016R had not shown significant dimer-specific nicking activity, the AP activity appeared to be retained at ~20% that of wild type. The other mutants, H034K, H056I, and H107K, retained 85, 65, and 51% of their AP activity, respectively.

$AgNO_3$ Inhibition of Dimer-Specific Nicking Activity. Previous studies in our laboratory have shown that the addition of Hg^{2+} and Ag^{+} to endonuclease V completely inhibits the two catalytic activities while nontarget DNA binding and pyrimidine dimer specific binding was retained (Prince et al., 1991). The single cysteine residue at position Cys78 has been demonstrated to be required for this metal binding since site-directed mutagenesis studies at this position (C078T and C078S) yielded enzymes which were catalytically active but were no longer inhibited by these metals (Prince et al., 1991). We also wished to determine whether histidine residues might contribute to this binding, and thus we tested whether Ag^{+} could effectively inactive the dimer-specific nicking activity of the histidine mutant proteins. All active histidine mutant proteins were completely inactivated by reaction with 2.5 mM $AgNO_3$ (data not shown). These results are consistent with the interpretation that Cys78 is primarily responsible for the metal binding.

Further Oligonucleotide Site Directed Mutagenesis at His16. The results shown in the previous sections concerning His16 showed that the H016R enzyme was unable to confer enhanced UV resistance to AB2480, probably because the mutant enzyme was deficient in pyrimidine dimer specific glycosylase activity, pyrimidine dimer specific complete incision activity, and apurinic lyase activity. These results raised the possibility that His16 may be associated with the catalytic activities of wild-type endonuclease V and thus additional mutations were produced at this codon by site-directed mutagenesis. The three additional changes were as follows: His16 to Lys16 (H016K), His16 to Tyr16 (H016Y), His16 to Thr16 (H016T). All mutations were confirmed by DNA sequence analysis. The mutant genes were subcloned; their orientation was confirmed and transformed into *E. coli* AB2480.

UV Survival of His16 Mutations. The colony-forming ability of *E. coli* AB2480 cells which harbor the plasmid producing wild-type or mutant endonuclease V was evaluated after challenges with increased doses of UV light. Unlike the H016R mutant enzyme, the H016K, H016Y, and H016T mutants all conferred enhanced UV resistance to the recipient cells albeit to levels less than that for the wild-type enzyme (Figure 6). These data suggest that catalytically active enzymes were produced whether amino acid 16 was lysine, tyrosine, or threonine. Thus, it is unlikely that His16 is directly involved in the catalytic nicking reactions of endonuclease V.

Enzymatic Activities of H016Y. In order to prove that the enhanced UV survival of *E. coli* harboring the plasmid expressing H016Y could be attributed to the retention of some pyrimidine dimer specific nicking activity, the H016Y enzyme

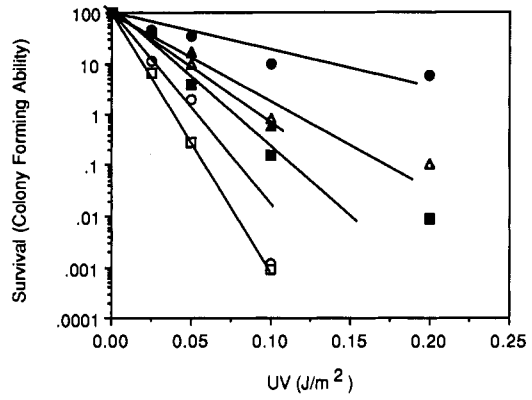

FIGURE 6: Colony-forming ability of UV-irradiated DNA repair deficient *E. coli* containing *den*V$^+$, *den*V$^-$, and His16 mutant plasmids. The experimental procedures were performed as described under Materials and Methods. (○) pGX2608 (*den*V$^-$); (●) pGX2608-16 (*den*V$^+$); (□) H016R; (■) H016K; (△) H016T; (▲) H016Y.

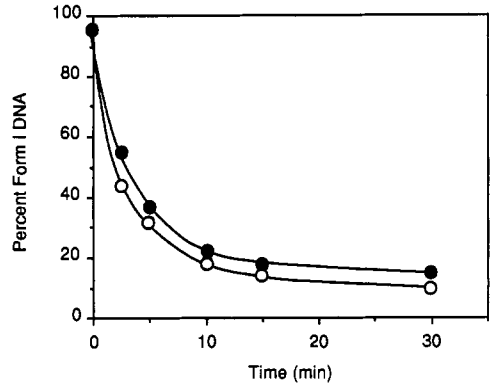

FIGURE 7: H016Y endonuclease V pyrimidine dimer specific nicking assay. ^{3}H-pBR322 DNA was UV-irradiated to introduce 25 pyrimidine dimers per molecule. The DNA was diluted into either 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, 1 mM EDTA, and 10% (v/v) ethylene glycol or 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 10% (v/v) ethylene glycol and the mixture prewarmed at 37 °C. Purified H016Y endonuclease V (15 ng per assay point) was added to each reaction mixture, and aliquots were removed at 2.5, 5, 10, 15, and 30 min. Reactions were terminated and processed as described under Materials and Methods. (○) 25 mM NaCl reaction conditions; (●) 100 mM NaCl reaction conditions.

was purified by single-stranded DNA–agarose chromatography, Sephadex G100 gel filtration chromatography, and heparin–agarose affinity chromatography. The concentration of enzyme was determined by Western blot analyses.

Kinetic pyrimidine dimer specific nicking assays were performed by using conditions in which the wild-type enzyme would function processively (25 mM NaCl) and distributively (100 mM NaCl) (Figure 7). When 15 ng of H016Y per assay was used, the rate of loss of form I DNA was the same at both 25 and 100 mM. However, this amount of activity represents a retention of only ~10% the activity of wild-type endonuclease V. In addition, at neither salt concentration did any form III DNA accumulate (data not shown). These results may suggest that the H016Y mutant locates pyrimidine dimers by a distributive mechanism even at low salt concentrations, thus accounting for the lack of accumulation of linearized DNA. Although this is one interpretation for these data in the case of enzymes which have a significantly lowered specific activity, a strict interpretation of data on target site location is difficult due to large amounts of enzyme which must be added to observe significant nicking activity. This reduced pyrimidine dimer specific nicking activity probably is the reason that cells expressing the mutant protein do not fully enhance the level of UV survival.

Kinetic apurinic DNA nicking assays were also performed comparing wild-type enzyme and H016Y. This mutant maintains ~25% apurinic nicking activity relative to that of wild-type enzyme (data not shown).

Discussion

The objectives of this study were to evaluate the roles that each of the four histidine residues within endonuclease V might have both on nontarget DNA binding and on the two catalytic activities of the enzyme. We chose to assess the importance of these residues by first creating mutations within the structural gene of endonuclease V and then by expressing and purifying these altered gene products.

None of the histidine residues within endonuclease V appear to directly participate in either the pyrimidine dimer specific glycosylic bond scission or the apurinic phosphodiester bond scission. The supporting evidence for this conclusion is as follows: a variety of conservative and sometimes radical amino acid substitutions for the histidines are catalytically functional. In this regard, mutants which were made at the His16 position displayed the greatest alterations in activity as compared to the wild-type enzyme. The first altered enzyme which was created at position 16, H016R, was shown to be inactive in the pyrimidine dimer specific glycosylase activity, and thus it was not possible to evaluate the phosphodiester bond scission activity using pyrimidine dimers as a substrate. However, when apurinic DNA was used as a substrate, the activity of this mutant was also severely reduced. Thus, it appears that the inability of H016R to enhance the UV survival of repair-deficient cells was most likely due to an inability to incise DNA at pyrimidine dimer sites. Although these initial data suggested that His16 may be an active-site amino acid, additional amino acid substitutions at His16 gave rise to mutant enzymes which still retained some dimer-specific nicking activity and could enhance UV survival in repair-deficient cells. No evidence exists that any of the other histidine residues directly participate in catalysis or are located within a portion of the enzyme that cannot accept significant amino acid alterations.

The predominant effect that mutations in the histidine residues had on the enzyme was to alter the affinities of the mutant enzymes for nontarget DNA. The mutants H034K and H107K both displayed wild-type levels of dimer-specific nicking activities and showed an in vivo enhanced UV survival that was indistinguishable from that conferred by the wild-type enzyme. However, the salt concentration at which nontarget DNA could no longer effectively compete for the enzyme was shifted from ~40 mM for the wild type to ~60 and 110 mM for H107K and H034K, respectively.

In addition, it was interesting to note that although the T4 endonuclease V did not display a modulation in the processivity of the nicking reaction as a function of pH as had been previously shown for the *M. luteus* UV endonuclease, the processivity of the wild-type T4 enzyme appears to be somewhat less than that of the *M. luteus* endonuclease (Figure 6). This result might have been predicted from previous data in which the *M. luteus* UV endonuclease does not change from a processive to a distributive target search mechanism until ~80–100 mM monovalent salt (Hamilton & Lloyd, 1989) while the salt concentration at which the transition occurs for endonuclease V has been shown to be ~40 mM monovalent salt (Gruskin & Lloyd, 1986). As revealed by the distribution of single-stranded DNA fragment sizes in Figure 7, the mutant enzymes H034K and H107K appear to have increased the processivity of the wild-type enzyme, so that the single-stranded

DNA fragment distribution was very similar to that of the *M. luteus* UV endonuclease.

The ability to utilize site-directed mutagenesis to increase the monovalent salt concentration over which endonuclease V can function processively has been demonstrated at other locations within the structural gene (Nickell & Lloyd, 1991). In those studies, mutations were engineered into a putative α-helix which may interact with the major groove of DNA so that basic residues were substituted at neutral positions in the enzyme. Several changes produced catalytically wild-type enzymes which also displayed enhanced affinity for nontarget DNA. Thus, the phenotypes of all of these mutant enzymes are very similar.

The data concerning the mutant H056I indicate that this alteration may be very complex to interpret. The majority of the data (kinetic pyrimidine dimer specific nicking assays at both low and high salt and the analysis of the distribution of single-strand breaks) suggest that this mutant functions by way of a distributive search mechanism even at a low salt concentration. However, the experiment in which unirradiated DNA was used as a competitor revealed that the presence of a 10-fold excess of unirradiated DNA significantly inhibited the enzyme's nicking of irradiated plasmid from 10 to 110 mM NaCl. These data are generally interpreted to mean that the enzyme is locating target sites by a processive mechanism at low salt and continues in a one-dimensional search throughout the concentrations of salt which were tested. However, since this interpretation of these data is in conflict with the other direct measurements of processive versus distributive search mechanisms, could there be an alternative explanation for the data produced by the competition experiments? In other data from this laboratory, Nickell and Lloyd (submitted for publication) have tested whether wild-type endonuclease V functions as a protein dimer. Model-building experiments suggested that protein–protein interactions might occur within identical putative α-helices (amino acid residues 54–62) between separate endonuclease V molecules. Directed mutations in the putative α-helix (54–62) have been shown to affect protein–protein interaction and thereby indirectly affect the competition experiments with nontarget DNA. Thus, it appears that protein–protein interactions are important for the DNA scanning. The reduced scanning of the H056I enzyme may also be a manifestation of a disruption in the protein–protein interaction.

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