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Articles

Site-Directed Mutagenesis of the T4 Endonuclease V Gene: Role of Lysine-130†

Adrian Recinos III‡ and R. Stephen Lloyd*,§

Departments of Microbiology and Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT: The DNA sequence of the bacteriophage T4 *den*V gene which encodes the DNA repair enzyme endonuclease V was previously constructed behind the hybrid λ promoter O_LP_R in a plasmid vector. The O_LP_R-*den*V sequence was subcloned in M13mp18 and used as template to construct site-specific mutations in the *den*V structural gene in order to investigate structure/function relationships between the primary structure of the protein and its various DNA binding and catalytic activities. The Lys-130 residue of the wild-type endonuclease V has been postulated to be associated with its apurinic endonuclease (AP-endonuclease) activity. The codon for Lys-130 was changed to His-130 or Gly-130, and each *den*V sequence was subcloned into a pEMBL expression vector. These plasmids were transformed into repair-deficient *Escherichia coli* (*uvr*A *rec*A), and the following parameters were examined for cells or cell extracts: expression and accumulation of endonuclease V protein (K-130, H-130, or G-130); survival after UV irradiation; dimer-specific DNA binding; and kinetics of phosphodiester bond scission at pyrimidine dimer sites, dimer-specific *N*-glycosylase activity, and AP-endonuclease activity. The enzyme's intracellular accumulation was significantly decreased for G-130 and slightly decreased for H-130 despite normal levels of *den*V-specific mRNA for each mutant. On a molar basis, the endonuclease V gene products generally gave parallel levels of each of the catalytic and binding functions with K-130 > H-130 > G-130 ≫ control *den*V⁻. A surprising exception to this trend was that G-130, while low in dimer-specific binding capacity, demonstrated an AP-endonuclease function several times as efficient as that of the wild-type enzyme. Overall, these results suggest that the alterations of Lys-130 chiefly compromise the ability of the mutant enzymes to bind DNA at dimer sites. Dimer-specific binding, however, appears not to be required for the AP-endonuclease activity.

The product of the bacteriophage T4 *den*V gene is a DNA repair enzyme, endonuclease V, which functions in the removal of UV-induced pyrimidine dimer photoproducts. The enzyme performs the initial strand scission in the repair of these lesions by mechanisms which involve four sequential actions: (1) a processive scanning process which monitors DNA for the presence of dimers; (2) a DNA binding function specific for dimer sites; (3) a pyrimidine dimer–DNA glycosylase (PD–glycosylase)[1] activity which cleaves the *N*-glycosyl bond be-

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‡ Department of Microbiology.
§ Department of Biochemistry.

[1] Abbreviations: PD–glycosylase, pyrimidine dimer–DNA glycosylase; AP, apurinic or apyrimidinic; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; RF, replicative form; kDa, kilodalton(s).

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tween the 5′-pyrimidine of a dimer and the corresponding deoxyribose; and (4) an apurinic/apyrimidinic endonuclease activity which finally hydrolyzes the phosphodiester bond immediately 3′ to the apyrimidinic site (Gordon & Haseltine, 1980; Lloyd et al., 1980; Radany & Friedberg, 1980; Seawell et al., 1980b; McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981; Nakabeppu et al., 1982; Ganesan et al., 198; Gruskin & Lloyd, 1986). The T4 *den*V gene has been stably cloned in plasmid constructs, and the expression of wild-type endonuclease V has been studied in DNA repair-deficient *Escherichia coli* (Valerie et al., 1985; Chenevert et al., 1986; Recinos & Lloyd, 1986; Recinos et al., 1986). The present studies concern the construction and the functional effects of site-directed mutations in the *den*V structural gene. Alterations were made which result in specific amino acid substitutions in the native repair enzyme. The mutant endonuclease V proteins were then expressed in *E. coli* and analyzed with regard to enzyme function.

Genetic and physiochemical data indicate that the dimer recognition and catalytic functions of endonuclease V may be associated with two distinct domains within the single polypeptide of the enzyme, since the PD–glycosylase and AP-endonuclease activities were shown to be differentially affected by certain T4 phage mutations (Minderhout et al., 1974; Sato & Sekiguchi, 1976; Seawell et al., 1980b; Valerie et al., 1984). The two activities of purified endonuclease V were demonstrated to possess different optimas for pH and salt concentration and different degrees of thermolability (Seawell et al., 1980b; Nakabeppu & Sekiguchi, 1981; Nakabeppu et al., 1982). Alternatively, these two catalytic activities may be determined by one active center which varies in dependence on a specific conformation of this center (Nakabeppu & Sekiguchi, 1981).

The selection of the amino acid sequences which affect the catalytic activities of the enzyme was guided by previous studies on the AP nicking activities of simple tripeptides such as Lys-Trp-Lys and Lys-Tyr-Lys (Behmoaras et al., 1981a,b; Pierre & Laval, 1981). It was shown that these tripeptides could efficiently bind and cleave DNA at AP sites. Examination of the endonuclease V amino acid sequence reveals an atypical region near its carboxy terminus (centered at Lys-130) which is rich in aromatic and basic residues, similar in nature to these tripeptides. Thus, we have altered the Lys-130 position in order to test the involvement of this residue in endonuclease V mediated glycosylic and phosphodiester bond scission.

In the following paper (Stump & Lloyd, 1988), the role of the aromatic amino acids which flank this Lys residue has been investigated to examine their role in the pyrimidine dimer-specific binding activity of endonuclease V. These aromatic amino acids were postulated to interact with regions of distortion in double-stranded DNA at the sites of pyrimidine dimers and thus correctly align this Lys-130 residue for DNA breakage reactions. The interpretation of results for these mutant endonuclease V molecules is that the carboxy-terminal portion of the endonuclease V is associated with pyrimidine dimer-specific binding but does not interfere with the incision activity on apurinic DNA.

Experimental Procedures

DNA Constructions and Oligonucleotide Site-Directed Mutagenesis. The *den*V structural gene was previously reconstructed in an *E. coli* expression vector (Recinos & Lloyd, 1986; Recinos et al., 1986). The promoter/operator, structural gene, and transcription terminator sequences were subcloned into M13mp18 RF (mp18-O_LP_R-*den*V), and phage were propagated in *E. coli* UT481 (constructed by Cynthia Lark, University of Utah, Salt Lake City, UT). Single-stranded DNA was prepared from phage essentially by the methods of Zoller and Smith (1983).

DNA oligonucleotides were designed from published *den*V sequences (Radany et al., 1984; Valerie et al., 1984), synthesized, and purified as previously described (Lloyd et al., 1986). The oligonucleotides were 21-mers which were complementary to the *den*V sequence from codon positions 127 through 133 except at the position of Lys-130. Here, alterations were made to specify His-130 (CAT) and Gly-130 (GGT). Both of these codons are preferentially utilized in highly expressed *E. coli* genes (Grantham et al., 1981).

Following a directed mutagenesis, plaques containing M13mp18-O_LP_R-*den*V phage with the desired mutation were selected by differential hybridization using the ^{32}P-end-labeled mutagenic 21-mer as a probe (Benton & Davis, 1977; Recinos & Lloyd, 1986). The positive phage were plaque purified, and after the preparation and isolation of the double-stranded mutant RF, the desired *den*V alteration was further confirmed by dot blot and Southern blot analyses. The wild-type and mutant O_LP_R-*den*V-t_{4S} inserts were then moved from the mp18 RF recombinants to pEMBL vectors (Dente et al., 1985) for expression in *E. coli*, generating pEMBL-*den*V$^+$-K-130, pEMBL-*den*V-H-130, and pEMBL-*den*V-G-130.

Intracellular Accumulation of Endonuclease V Proteins. The above-mentioned pEMBL constructs were each transformed into *E. coli* AB2480 (*uvr*A$^-$ *rec*A$^-$) and again selected by hybridization to specific probes. AB2480 cells harboring plasmids for the expression of wild-type and mutant endonuclease V proteins (henceforth denoted proteins K-130, H-130, and G-130) were routinely grown at 30 °C to stationary phase in LB medium supplemented with 100 μg/mL ampicillin. In all expression and activity studies, cells containing negative control plasmids pGX2608 (which has no *den*V insert) or pEMBL-19(+) (which has no O_LP_R-*den*V-t_{4S} insert) or pGX2608-19-*den*V$^-$ (which has the *den*V structural sequence inserted in the incorrect orientation relative to expression from O_LP_R) (Recinos & Lloyd, 1986) were cultured in parallel.

Cells were harvested and equilibrated for cell number by optical density, and crude cell lysates were evaluated for the accumulation of immunoreactive endonuclease V by Western blot analyses (Laemmli, 1970; Towbin et al, 1979; Burnette, 1981; Higgins & Lloyd, 1987; Lloyd & Augustine, 1986). Total intracellular protein levels were determined by using the BCA protein assay (Pierce Chemical Co., Rockford, IL). For antibody-independent measurements of endonuclease V accumulation, the three pEMBL-*den*V constructs and a negative control plasmid were each transformed into *E. coli* X1411 which is a minicell-producing strain. Minicells were purified and radiolabeled with ^{35}S and ^3H amino acids (Roozen et al., 1971, with modifications; P. Arthur, personal communication), and proteins were analyzed by electrophoresis on SDS–polyacrylamide gels in conjunction with autoradiography and densitometry.

Cell Extracts Containing Endonuclease V Proteins. AB2480 cells expressing the various endonuclease V proteins were grown to stationary phase ($A_{600} \approx 4$), pelleted, and resuspended to $A_{600} = 8.0$ in buffer containing 100 mM KCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10% (v/v) ethylene glycol. Extracts were prepared from 2.5 mL of each of these solutions by thorough sonication on ice. After cell debris was removed by centrifugation, the supernatants were stored at 4 °C as stock enzyme-containing cell extracts. Molarities of repair enzyme in cell extract were determined

from data of total cellular protein, Western blot analyses, and minicell analyses.

UV Survival. In vivo UV dose–response data measured as colony-forming ability were performed as previously described (Recinos et al., 1986).

Dimer-Specific Nicking Activity. The ability to make single-stranded nicks in vitro in UV-irradiated DNA was monitored as a measure of complete endonuclease V function. Assays were performed basically as previously described (Lloyd et al., 1980; Lloyd & Augustine, 1986).

PD–Glycosylase Activity. The initial catalytic cleavage of endonuclease V at the site of a pyrimidine dimer is a glycosylic bond scission. Nakabeppu and Sekiguchi (1981) demonstrated that in short periods of in vitro reaction at pH 8.0, these sites accumulate to significantly higher frequencies than do sites of complete single-stranded nicks. The formation of alkali-labile sites in excess of single-stranded nicks was monitored by two assays. In addition to monitoring the reaction for complete incision as previously described, we added 1 N NaOH to the remaining half of the reaction, yielding a final concentration of 0.167 N NaOH. This mixture was incubated at 37 °C for 1 h to convert AP sites remaining from the initial reaction to single-strand breaks. The hydrolysis reaction was neutralized with 1 N HCl and kept at room temperature for 75 min. Following electrophoresis, gel bands were processed for scintillation counting as previously described.

AP-Endonuclease Activity. Unirradiated but acid-depurinated pBR322 DNA (Lindahl & Andersson, 1972; Lindahl & Nyberg, 1972; Lloyd et al., 1978) was prepared as substrate in order to examine the AP nicking activity of endonuclease V. A batch mixture of plasmid DNA which contained, for each assay of AP-endonuclease activity, 1 μg of unlabeled pBR322 and 0.25 μg of ^{3}H-pBR322 was concentrated by ethanol precipitation. The dry DNA precipitate was resuspended in 10 mM trisodium citrate (pH 4.0), 1.5 mM Tris-HCl (pH 8.0), and 105 mM NaCl, incubated at 65 °C for 5 min, and neutralized with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The depurinated plasmid DNA was then stored at −20 °C as an ethanol precipitate, and for use, it was resuspended in 10 mM Tris-HCl (pH 8.0) and 0.2 mM EDTA. Enzyme reactions, electrophoresis, and scintillation counting for examining AP-endonuclease activity with this DNA substrate were conducted in the same manner in which dimer-specific nicking was monitored.

Binding to UV-Irradiated DNA. The capacity of endonuclease V proteins to retain UV-irradiated DNA to a nitrocellulose filter was assayed by methods developed by Seawell et al. (1980a) with slight modifications. In brief, ^{3}H-pBR322 was UV-irradiated for a total dose of 200 J/m^2 (yielding approximately 12 dimers per molecule), and a 20-μL aliquot containing a limiting amount of cell extract (enzyme) was added to the 0.25 μg of ^{3}H-pBR322 in a standard reaction mixture on ice. The remainder of the analysis was conducted as per Seawell et al. (1980a). Control assays to correct for nonspecific retention of ^{3}H cpm were included. Dimer-specific binding was calculated by normalizing to 100% the amount of DNA retention that resulted from saturating concentrations of purified T4 endonuclease V (0.15–2.9 μM) and then expressing filter retention due to the extract proteins as a percentage of this maximum.

Results

Accumulation of Endonuclease V Proteins in Cells Harboring denV Constructs. The DNA manipulations involving site-directed mutagenesis and subcloning procedures generated three vectors for the expression of wild-type and mutant endonuclease V (K-130, H-130, and G-130). Each of the mutant structural gene constructs were completely sequenced to verify that only the predicted changes had been made. When these plasmids were transformed into *E. coli* AB2480 and accumulations of the wild-type and mutant endonuclease V proteins were evaluated by Western blot analyses, it was observed that the single-codon substitutions at position 130 resulted in altered levels of accumulation of immunoreactive enzyme (Figure 1A). Densitometric scans of typical blots indicated that K-130, H-130, and G-130 accumulated to 0.49%, 0.43%, and 0.09%, respectively, of total cellular proteins.

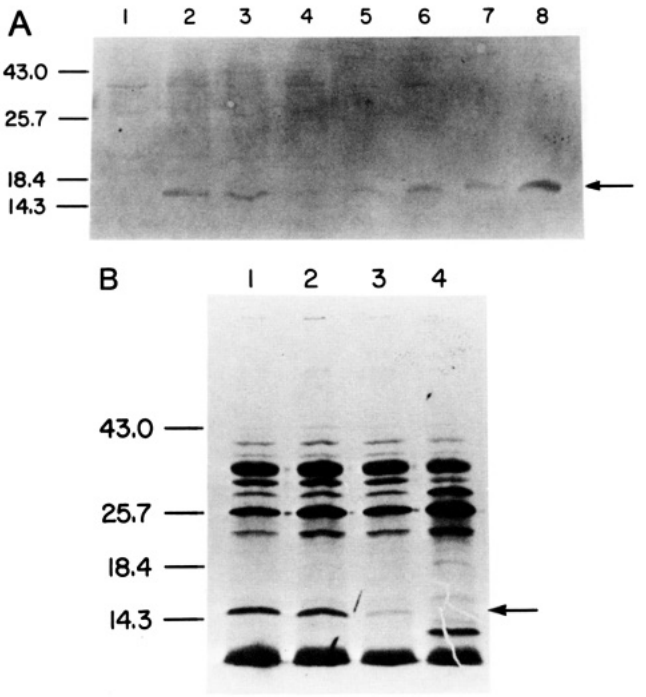

FIGURE 1: (A) Western blot analysis of endonuclease V proteins accumulated in *E. coli* AB2480 (*uvr*A *rec*A) expressing pEMBL-*den*V constructs. Rabbit anti-endonuclease V antibodies were used for detection. The *den*V proteins examined were K-130 (lane 2), H-130 (lane 3), and G-130 (lane 4). Cross-reactive backgound peptides (lane 1) are illustrated by a transformant harboring a negative control *den*V$^-$ plasmid. Samples loaded contained 40 μg of total lysate proteins. Purified endonuclease V standards contained 40, 100, 200, and 500 ng of enzyme protein (lanes 5–8). Molecular weight standards (×10^{-3}) were as indicated at the left. The arrow denotes the position of endonuclease V at 16 kDa. (B) Accumulation of radiolabeled endonuclease V proteins in *E. coli* X1411 minicells. Minicells produced by transformants harboring pEMBL-*den*V constructs were purified from overnight cultures by centrifugation. Aliquots of 3 × 10^8 minicells were radiolabeled for 1 h at 30 °C with 50 μCi of L-[^{35}S]methionine (specific activity 1100 Ci/mM) and 10 μCi of L-[^{3}H]leucine (specific activity 140 Ci/mM), washed, and prepared for electrophoresis through a 0.75-mm-thick 11% SDS–polyacrylamide separating gel. Following electrophoresis, the gel was fixed, processed for fluorography, dried, and exposed to X-ray film. Proteins examined were encoded by pEMBL constructs expressing K-130 (lane 1), H-130 (lane 2), G-130 (lane 3), and the uninterrupted (no *den*V insert) *lac*Z α-peptide (lane 4). Molecular weight standards (×10^{-3}) were as indicated at the left. The arrow denotes the position of endonuclease V.

In order to exclude the possibility that the variations in detection by Western blot analysis merely reflected epitopic changes in the mutant enzymes which altered the affinity of the endonuclease V antibodies, intracellular accumulations of the wild-type and mutant endonuclease V proteins were examined in *E. coli* minicells. Autoradiographs of plasmid-encoded ^{35}S- and ^{3}H-labeled protein bands (Figure 1B) demonstrated that relative accumulations of K-130 (lane 1), H-130 (lane 2), and G-130 (lane 3) were similar to those which were observed by Western blot analysis. The identities of the three

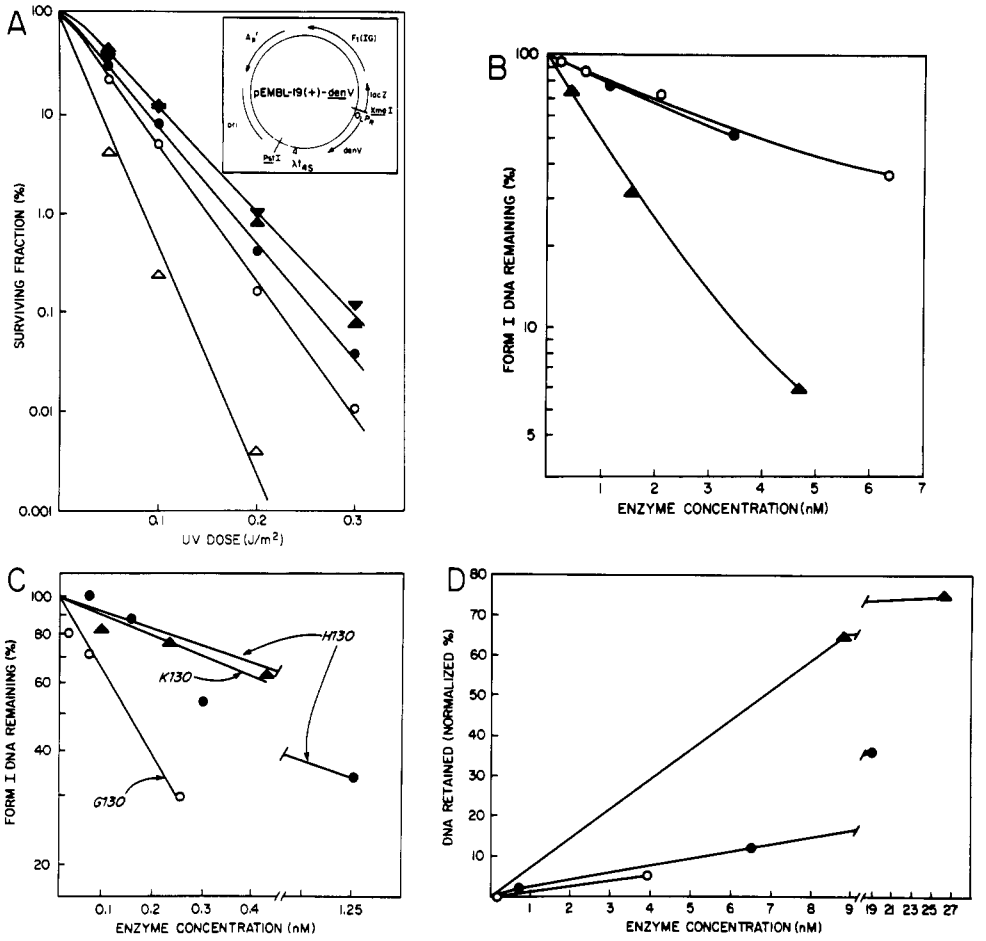

FIGURE 2: (A) Survival following UV irradiation of *E. coli* AB2480 (*uvr*A *rec*A) containing *den*V plasmid constructs. Surviving fraction of cells plated and irradiated is plotted for cultures transformed with pGX2608-16-*den*V (▲), pEMBL-*den*V⁺-K-130 (▼), pEMBL-*den*V-H-130 (●), pEMBL-*den*V-G-130 (○), and the negative control pGX2608-19-*den*V⁻ (△). Inset: Schematic diagram of pEMBL constructs expressing *den*V enzymes. (B) Kinetics of endonuclease V proteins in producing pyrimidine dimer-specific single-strand breaks in DNA. The assay measured complete enzymatic activity for limiting concentrations of each protein. Plotted percentage values which illustrate losses of initial form I DNA in the reactions were corrected to exclude non-dimer-specific nicking and form I DNA which did not contain dimers. Enzyme concentrations of K-130 (▲), H-130 (●), and G-130 (○) are final reaction concentrations and are based on accumulations in *E. coli* AB2480 cell extracts. (C) Kinetics of endonuclease V proteins in producing single-strand breaks at apurinic sites in DNA. The assay measured AP-endonuclease activity for limiting concentrations of each protein. Resultant curves which indicate form I DNA losses in the reaction are fitted by using standard linear regression techniques. Percentages of form I DNA remaining were corrected to exclude non-AP-specific nicking, nicking due to background AP-endonuclease activities, and form I DNA which did not contain AP sites. Enzyme concentrations of K-130 (▲), H-130 (●), and G-130 (○) are final reaction concentrations and are based on accumulations in *E. coli* AB2480 cell extracts. (D) Kinetics of pyrimidine dimer-specific DNA binding by endonuclease V proteins. The standard binding assay of Seawell et al. (1980a) was used to quantitate amounts of UV-irradiated ^{3}H-pBR322 DNA retained on nitrocellulose filters due to DNA binding by limiting concentrations of protein. Dimer-specific binding was obtained by correcting for retention of unirradiated DNAs. Normalized percent DNA retained was calculated by equating the dimer-specific retention observed with saturating final concentrations of purified T4 endonuclease V (2660 cpm, 45% of total plasmid cpm) to 100%. Enzyme concentrations of K-130 (▲), H-130 (●), and G-130 (○) are final concentrations in the 3-min binding reaction.

bands migrating at the position of endonuclease V were confirmed by comparison with proteins expressed by a pEMBL-(19+) vector which contained no cloned insert (lane 4). The two β-lactamase peptides (and some minor contaminating host proteins) were again visualized at the higher molecular weights, but the lower band at the position of endonuclease V has been replaced by the 13-kDa truncated β-galactosidase protein which is expressed by the vector alone. Densitometric scans of this radiograph showed that for a constant number of minicells, H-130 accumulation was 83% of that for K-130, while G-130 accumulated to only 16% of the level for the wild-type enzyme. In addition, steady-state mRNA levels were determined by slot blot analyses using a 5′ *den*V-specific oligonucleotide as probe. Transcription levels from the three *den*V genes were comparable, suggesting that the steady-state accumulation of *den*V-specific message was not the limiting step in protein accumulation. These findings of decreased mutant protein accumulation are very consistent with data presented in the following paper (Stump & Lloyd, 1988) in which mutant enzymes (changes at Tyr-129,131) also accumulated to only 15–20% that of wild-type.

Enhancements of UV Survival. Transformation of repair-deficient *E. coli* AB2480 (*uvr*A *rec*A) with the K-130 expression plasmid afforded UV survival increments which were equal to those seen for a parent construct, pGX2608-16-*den*V⁺ (Figure 2A, closed triangles). These enhancements relative to cells harboring a negative control plasmid, pGX2608-19-*den*V⁻ (open triangles), were essentially the same as those previously reported (Recinos et al., 1986). Plasmids which expressed the mutant proteins H-130 (closed circles) and G-130 (open circles) exhibited intermediate capacities to rescue the repair-defective host cell phenotype.

Catalytic Activities of Endonuclease V Proteins. The relative efficiencies of K-130, H-130, and G-130 proteins in catalyzing complete dimer-specific incisions in form I DNA are shown in Figure 2B. At very low enzyme concentrations, only a small percentage of the initial substrate is nicked in 30 min, and the reactions display a first-order dependence on

Table I: Total PD–Glycosylase Activity: Sum of Alkali-Labile AP Sites Formed and Complete Phosphodiester Bond Scissions

enzyme	concn (nM)	form I DNA remaining after initial dimer-specific complete nicking reaction (%)[a]	form I DNA remaining after initial reaction followed by hydrolysis at AP sites (%)[a]	ratio of remaining form I DNAs
K-130	0.4	64	31	2.1
	1.6	32	21	1.5
H-130	0.3	99	76	1.3
	1.1	88	53	1.6
	10.4	36	22	1.6
G-130	0.2	99	83	1.2
	2.1	81	70	1.2

[a] Data are corrected to exclude nonspecific nicking and form I DNA not containing dimers.

enzyme concentration. The slopes of the curves depicted in this plot indicated that K-130 (closed triangles) performed the complete in vitro endonuclease V reaction 4 times as efficiently as did H-130 (closed circles) or G-130 (open circles). The latter two proteins did not deviate significantly in these assays.

Pyrimidine dimer-specific glycosylase activity was monitored in a procedure which quantitated the formation of AP sites which were not converted to phosphodiester scissions (Table I). Under the pH conditions used, K-130 extracts produced the expected (Nakabeppu et al., 1982) excess number of AP sites relative to single-strand nicks (last column of the table). The ratio of complete incision versus glycosylase activity was markedly decreased for G-130 and slightly decreased for H-130. Thus, the two mutant proteins again showed comparable and much diminished capacities to make complete incisions at dimer sites. Although G-130 produced the fewest total AP sites (i.e., complete nicks plus alkali-labile sites), almost all of these sites were converted to breaks by the enzyme. H-130 appeared more like the wild-type K-130 in this comparison of relative levels of the two catalytic activities.

The capacities of these enzymes to produce single-strand breaks in DNA containing apurinic sites demonstrated large variations in the nicking efficiency of G-130 with respect to the other two enzymes (Figure 2C). K-130 (closed triangles) and H-130 (closed circles) gave similar reaction profiles while the curve for G-130 (open circles) indicated a reaction efficiency which was more than 4 times greater than that for the wild-type enzyme. It was also noted that extremely low enzyme concentrations provided appreciable AP endonuclease activity in these assays.

In total, the findings of the three assays of catalytic function indicated that the limiting or defective aspects of the mutant proteins H-130 and G-130 most probably involved not AP-endonuclease activity, as expected from previous reports, but rather some other mechanism(s) directed at dimer-specific DNA binding and/or the initial glycosylic cleavage.

Dimer-Specific DNA Binding. The assays of the capacity of each enzyme to bind UV-irradiated DNA demonstrated that H-130 and G-130 were markedly deficient in this regard in comparison to the wild-type protein (Figure 2D). Results of these experiments showed that while K-130 bound UV-irradiated DNA with relatively high affinity (closed triangles) and H-130 (closed circles) and G-130 (open circles) showed far less dimer-specific binding. The percent DNAs retained per nanomolar enzyme concentration were 7.2, 2.4, and 0.8 for K-130, H-130, and G-130, respectively. The magnitude of the variation in these results provides strong evidence that the major deficiencies of H-130 and G-130 with respect to total endonuclease V function lie in their reduced abilities to bind DNA at dimer sites. More subtle aspects of the functional analyses of the two mutant proteins are discussed below.

A summary of the results of the various assays employed in evaluating the enzymatic efficiencies of the endonuclease V proteins is provided in Figure 3. This plot collates the approximate relative levels of function pertinent to H-130 (striped bars), G-130 (solid bars), and the wild-type K-130 by normalizing the latter to 100% for each analysis.

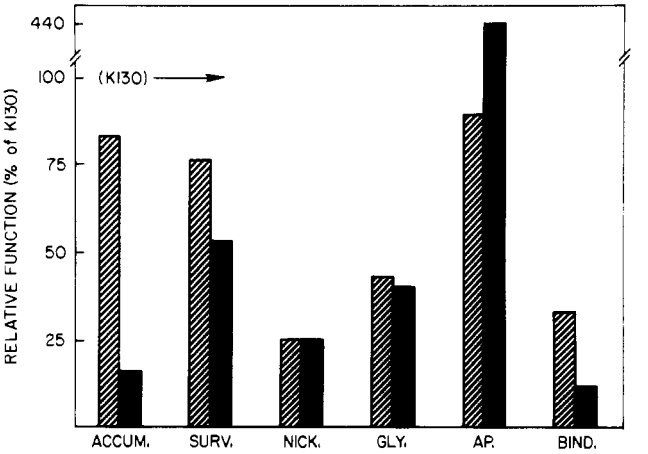

FIGURE 3: Summary of enzymatic efficiencies of endonuclease V proteins. Relative overall levels of function for *E. coli* AB2480 cells or cell extracts containing H-130 (striped bars), G-130 (solid bars), and wild-type K-130 (normalized to 100% for each assay). Base line 0% is equilibrated for any background levels in host cells or cell extracts. Abbreviations and basis for approximate levels are as follows: ACCUM., intracellular enzyme accumulation from Western blot, minicell, and total protein analyses (see Figure 1); SURV., UV survival from dose resulting in 0.1% survival of cells plated (see Figure 2A); NICK., complete dimer-specific nicking activity from initial velocity kinetics (see Figure 2B); GLY., total PD–glycosylase activity from initial velocity kinetics (see Table I); AP., AP-endonuclease activity from initial velocity kinetics (see Figure 2C); BIND., dimer-specific DNA binding from initial slope of filter retention curve (see Figure 2D).

Discussion

The site-specific alterations of the wild-type endonuclease V which were constructed and evaluated in this study lend several insights into the manner in which this multifunctional repair enzyme interacts with DNA. The amino acid substitutions at the position of the native lysine-130 perturb a region of the molecule which has received much speculative attention. These speculations have arisen chiefly from data concerning the chemistry of peptide–DNA interactions and the enzymatic phenotypes of T4 *den*V mutants with known sequence anomalies. The evaluations of the mutant enzymes presented here (and summarized in Figure 3) provide refinements to these previous postulations.

Endonuclease V G-130 exhibited a number of interesting and elucidative characteristics. This is the first reported instance of a mutant endonuclease V protein possessing full AP-endonuclease activity with concomitant and severe loss of PD–glycosylase and dimer-specific binding functions. This observation for G-130 is especially surprising because the latter

functions are markedly depressed while the former activity is elevated severalfold over levels found for the wild-type enzyme. Although this mutant protein accumulates to lower concentrations than do K-130 or H-130, its expression clearly enhances the UV survival of repair-deficient cells. Results indicating that low intracellular accumulations of a binding-defective enzyme (G-130) provide substantial enhancements in UV survival are seemingly contradictory. These observations become more understandable when analyzed with respect to pertinent findings for the wild-type enzyme. Since growth conditions can be adjusted such that the appearance of immunoreactive K-130 on Western blots is greatly reduced while the UV survival remains unaffected (Recinos et al., 1986), it appears that in vivo concentrations of wild-type endonuclease V are above that required for maximal levels of UV resistance.

The enzyme characteristics of G-130 clearly indicate that lysine-130 is not essential for phosphodiester cleavage at AP sites. It could be argued that the AP sites of depurinated DNA are not strictly identical with the AP sites which are formed by the initial PD–enzyme's glycosylase activity, in which the 5′-pyrimidine remains dimerized and in the vicinity of the reaction. Thus, given the "true" substrate, G-130 might not retain its enhanced AP-endonuclease efficiency. This possibility is unlikely though, because the PD–glycosylase assay demonstrated that while G-130 does not catalyze the formation of as many AP sites as the wild-type enzyme at the site of a pyrimidine dimer, most all of those which are formed are subsequently converted to single-stranded breaks. The large increment in G-130 AP-endonuclease activity over that of even the wild-type enzyme is reasonable since this catalysis does not require the presence of a dimer in the DNA before or during the actual incision. Thus, the removal of an important constraint which enables the enzyme to perform dimer-specific binding (i.e., the lysine at position 130) appears to allow the molecule to gain additional effectiveness in AP-endonuclease activity. In this regard, it is also of interest to note that when the two Tyr residues which flank this Lys are changed to Asn residues, the resultant enzyme retains nearly 100% of its AP nicking activity while losing all pyrimidine dimer-specific binding activity (Stump & Lloyd, 1988).

The less drastic amino acid substitution at position 130 gave rise to the H-130 protein and at least maintained an R group and the potential for a positive charge at this position. In some analyses (complete dimer-specific nicking, total glycosylic cleavages, and UV-irradiated DNA binding), the H-130 enzyme appeared essentially as defective as G-130. The major qualitative difference between the two mutant proteins was that H-130 was not observed to vastly enhance AP-endonuclease activity. In contrast, it demonstrated a small decrease in this function relative to the wild-type enzyme.

The AP-endonuclease characteristics of H-130 and G-130 are particularly interesting in comparison to the T4 *uvs-13* amber mutant, which when suppressed has been shown to possess partial (~15–20% relative to T4D) glycosylase activity but no AP-endonuclease activity (Nakabeppu et al., 1982). Suppression of the *uvs-13* mutation results in the substitution of serine for the tryptophan of the wild-type enzyme at position 128 (Valerie et al., 1984), and thus, Trp-128 appears to be critically involved in the AP-endonuclease function. Valerie et al. (1984) speculated that endonuclease V may make incisions at AP sites by mechanisms similar to those utilized by the tripeptides Lys-Trp-Lys and Lys-Tyr-Lys. It is clear that the endonucleolytic activity of these tripeptides is related to a stacking interaction between the central aromatic residue and nucleic acid bases flanking the AP site (Behmoaras et al., 1981a,b). It is also thought that the actual tripeptide-induced strand breakage occurs by β-elimiantion since the reaction chemistry conforms to this mechanism (Pierre & Laval, 1981). An ϵ-NH_2 group of one of the adjacent lysines in the tripeptide has thus been implicated in the cleavage mechanism. For the AP-endonuclease activity of endonuclease V, however, it is evident from the enzymatic character of G-130 (and to a lesser extent, H-130) that while Trp-128 may well be intrinsic to an effective AP-endonucleolytic reaction, the close positioning of Lys-130 in the primary sequence is dispensable. The possibility remains, of course, that the tertiary structure of the enzyme molecule places another lysine in "tripeptide-like" functional proximity to Trp-128. An X-ray crystal structure of the wild-type enzyme (in progress) should provide a guide to the identification of specific amino acids involved in its activities.

Previous reports of differential losses of the two endonuclease V catalytic activities, observed for enzymes encoded by phage T4 amber mutants and also for wild-type enzyme preparations under certain reaction conditions, have indicated a generally greater fragility of the AP-endonuclease relative to the PD–glycosylase (Nakabeppu & Sekiguchi, 1981; Nakabeppu et al., 1982). Thus, the activity for cleavage at AP sites consistently is reduced more extensively when different activities have been noted. The specific defects in H-130 and G-130, however, illustrate the reverse of this trend since both mutant proteins are more diminished for PD–glycosylase activity than for AP-endonuclease activity. These large reductions in glycosylase function for H-130 and G-130 are highly likely to be directly inherent to their similarly depressed capacities for dimer-specific DNA binding (since this binding would be expected to be required for the initial dimer-specific cleavage). These data which suggest that this portion of the enzyme is associated with dimer-specific binding are also in good agreement with the findings in the following paper (Stump & Lloyd, 1988) in which the aromatic residues surrounding the Lys-130 were changed to polar nonaromatic amino acids. Alterations in these flanking amino acids totally abolish pyrimidine dimer-specific binding. The loss of binding activity in those mutants was (as would be predicted) also associated with a complete loss in dimer-specific nicking activity. Together, these data strongly support a role for the C-terminal domain of endonuclease V in the recognition and binding to pyrimidine dimers.

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Registry No. T4 endonuclease V, 52227-85-7; apurinic endonuclease, 61811-29-8; lysine, 56-87-1; glycine, 56-40-6; pyrimidine dimer glycolase, 75302-33-9.

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