Structure/function analysis of the Ala¹¹⁶ → Lys¹²¹ region of endonuclease V by random targeted mutagenesis

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ABSTRACT

Endonuclease V is the product of the *denV* gene of bacteriophage T4 and is responsible for the recognition and repair of pyrimidine dimers due to UV irradiation of DNA. This is accomplished by a two-step mechanism involving incision at the site of the lesion followed by cleavage of the phosphate backbone. In order to better understand this molecule, and to validate our new mutagenesis procedure, we have constructed a series of random mutations within the region Ala¹¹⁶ → Lys¹²¹ using a random targeted mutagenesis procedure developed for this study. The results presented here suggest an important role for this region in the stabilization of the thymine dimer-containing substrate. These mutants also confirm a direct correlation between survival and both DNA binding and pyrimidine dimer-DNA glycosylase activity. No such correlation exists between survival and AP lyase activity. The results are consistent with the recently published X-ray crystal structure.

INTRODUCTION

Endonuclease V (EC 3.1.25.1) is a DNA repair enzyme encoded by the *denV* gene of bacteriophage T4. This enzyme recognizes and mediates the repair of the pyrimidine dimer photoproducts in DNA due to ultraviolet (UV)¹ irradiation. The enzyme accomplishes this by utilizing three distinct activities associated with the molecule: (i) DNA binding of pyrimidine dimer-containing DNA; (ii) a pyrimidine dimer DNA-glycosylase activity that cleaves at the 5'-thymine of the dimer; and (iii) an apurinic/apyrimidinic (AP) lyase (formally endonuclease) activity that cleaves at the phosphodiester bond 3' to the AP site (1, reviewed in 2). The *denV* gene has been cloned and sequenced (3, 4).

Results with T4 mutants have suggested that the two principle activities (DNA glycosylase and AP lyase) occur on the same molecule, but that, since these activities can be separated based on ionic strength, temperature and pH, these activities may lie in different regions of the molecule (5, 6). Until recently, little

direct data existed which could correlate one segment of endonuclease V with a particular activity. Initial observations that certain tripeptides with aromatic residues, such as those in the region Trp-Tyr-Lys-Tyr (residues 128 to 131), have AP lyase activity (7, 8) was used to speculate that the catalytic site of the enzyme resides in this area. Using site-directed mutagenesis, Lloyd and colleagues looked at changes in this region and their effects on enzyme activity. Their results show that mutations in this region resulted in either no effect or decreases in DNA glycosylase activity, which seem to correlate with DNA-binding activity. By contrast, there was no such pattern seen with AP-endonuclease. In one case AP lyase activity was increased as much as 400-fold despite decreases in both PD glycosylase and DNA-binding activities (9). In general, their results demonstrated an association of Lys¹³⁰ with DNA binding and endonuclease activity at AP sites (9), and Tyr^{129/131} with binding and endonuclease activity at PD sites (10), but overall that this region was not absolutely required for PD glycosylase or AP lyase activity. There were virtually no mutants in this region that did not have a profound effect on the enzyme. Interestingly, none of their mutations was able to confer an increase in DNA glycosylase activity. These results were confirmed by Ishida and coworkers looking at an additional seven mutations in the 128–132 region, showing a correlation between *in vivo* survival and *in vitro* PD glycosylase activity (11). The first evidence for the role of the amino terminus and not the carboxy terminus as integral to the catalytic site was shown by Dowd and Lloyd (12) demonstrating the role of Arg³. The role of the amino terminus was confirmed by Schrock and Lloyd (13). Most recently, Morikawa et al. have solved the X-ray crystal structure for endonuclease V which puts significant emphasis on the role of Glu²³ in catalytic activity as well as regions in the amino terminus, including Arg³ (14,15).

In an attempt to continue the study of the structure/function relationship of this enzyme, we utilized a newly developed procedure for random targeted mutagenesis that can be used with any plasmid. This procedure is a modification of procedures incorporating polymerase chain reaction (PCR) amplification of a region of DNA with the use of a combination of mutagenic

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and nonmutagenic primers(16–18). To validate this procedure most efficiently, the region Ala¹¹⁶ → Lys¹²¹ was chosen for investigation.

MATERIALS AND METHODS

Materials

Cell lines

E. coli strain AB2480 (*uvrA6*, *recA13*, [F'*lacI*^qZ⁺Y⁺, *proAB*]) (19); strain BMH 71-18 mut S (Promega, Madison, WI) (20), (*thi*, *supE*, Δ(*lac-proAB*), [*mutS*::Tn10] [F', *proA*⁺B⁺, *laqI*^qZΔM15]) and strain JM109 (Promega) (21) (*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*(r⁻_k,m⁻_k), *relA1*, *supE44*, λ⁻, Δ(*lac-proAB*), [F'*traD36*,*proAB*,*lacI*^qZΔM15]) were used in this study.

Plasmids

For mutagenesis using Promega's Altered-Sites kit (see below), the plasmid pSelect-denV was made by cloning the SacI-Sall fragment from pGem-denV (made by cloning the ClaI fragment of pdenV-52 [19] into pGem7Z[f⁻]) into the SacI-Sall fragment of the phagemid pSelect-1 (Promega). For PCR mutagenesis and high level expression of Endonuclease V, the plasmid ptac-denV was constructed (Figure 1). This plasmid contains the IPTG-inducible *lac-trp* (*tac*) promoter (22) with strong stops in all three reading frames (23) and terminators (24) for proper processing of the transcript. The plasmid was constructed by ligating the filled-in *HinfI* fragment of pdenV-52 that contains the structural gene for *denV* (19) into the filled-in *NcoI* linearized ptac-1. Plasmid ptac-1 was made by filling-in the *EcoRI*-*HindIII* fragment from pK01(galK⁻) (25) and ligating into the *PvuII*-*HindIII* fragment of m13mp10 (containing the *lac* promoter/operator and the multicloning sites). The ~875 bp *PvuII*-*HindIII* fragment from this construct was ligated to the *PvuII*-*HindIII* fragment of pEX1 (24) that contains the three-phase translation stops and the *fd* phage terminators and recovers the ampicillin resistance gene to make pEX1(lac). The *tac* promoter element was obtained by addition of a synthetic *NcoI*-*BamHI* linker to *PvuII*-linearized ptac12 (22). This construct as well as the pEX1(lac) construct were cut with *EcoRI* and *BamHI* and ligated to form ptac-1.

Oligonucleotides

All oligonucleotides were synthesised using an Applied Biosystems Model 380 Synthesizer by the Macromolecular Synthesis Facility of Temple University (see Table I for sequences). For those oligonucleotides containing random substitutions, the phosphoramidite mixtures were 'spiked' with ~6¼ % of each nonappropriate phosphoramidite, so that the final 'spiked' was 18¾ %. At this percentage, one can expect from 1 to 3 substitutions per molecule of synthesized oligonucleotide (26).

Methods

Mutagenesis procedures

Gapped-duplex mutagenesis

The Altered-Sites in vitro Mutagenesis system was utilized to make Gln¹²¹ and used according to the manufacturer's instructions. In this system, the *denV* gene was cloned into the vector pSelect-1, which contains the tetracycline resistance gene and a mutated ampicillin gene. After sequencing to confirm the mutation, the *XhoI*-*BamHI* fragment was subcloned into ptac-denV.

Figure 1. Map of ptac-denV (~3.2 kb).

Figure 2. Outline of PCR-mediated mutagenesis procedure.

Random targeted mutagenesis

Mutagenesis was performed using the over-expressing vector ptac-denV as a template for creating a population of random mutations within the *XhoI*-*BamHI* region of this vector. This region consists of the 3'-half of the *denV* gene (at the *XhoI* site) through the multi-cloning region of the vector (including the *BamHI* site). The procedure uses a combination of non-spiked and randomly spiked oligonucleotides and PCR amplification to create a random 'cartridge' that can be easily subcloned into the expression vector. An outline of the procedure is shown in Figure 2. In brief, two initial amplifications were carried out using the amplimers in Table I. Amplimers 1 and 4 include the cloning sites within the sequence. Amplimers 2 and 3 are complementary and contain random substitutions identified by boxes. Amplification was carried out using 100 ng of template, 1 μM primer (final), 200 μM each dNTP, Taq buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5mM MgCl₂, 0.01% gelatin and 0.1% Triton X-100) and 2.5 U Taq polymerase (Promega). Conditions for amplification were 30 cycles at 94°C/1 min; 48°C/2 min; 72°C/3 min. Products were analyzed on 2.0% agarose gels, and the products of the initial amplification were

Table I. Oligonucleotides

	PCR product size
Xho I	
Primer 1: 5'-GATAAGCTCGAGTTCTTACGT-3'	
Primer 2 ^a : 3'-TATAGTGTTCGAGCAAATCTACTTTTAAACGTGTT-5'	1/2 189 bp
Primer 3 ^a : 5'-ATATCACAAGCTCGTTTAGATGAAAAAATTGCACAA-3'	
Primer 4: 3'-CCTAGGAGATCTCAGCTGGACGTC-5'	3/4 135 bp
BamH1	
Final product size (1/4)	324 bp

^a Highlighted bases are randomized.

purified on PrimeErase columns (Stratagene, La Jolla, CA) and concentrated by precipitation in ethanol. A second round of amplification was done using 1 μ l of each of the products from the initial amplification, purified as before, cut with *Xho*I and *Bam*H1 and ligated into *Xho*I-*Bam*H1 cut ptac-denV, and transformed first into BMH 71-18 mutS. Transformation was done in culture overnight with ampicillin (100 μ g/ml), minilysates were prepared (27). Samples were treated with RNase and then transformed again into AB2480. Individual colonies were picked and DNA was isolated as before and sequenced by dideoxy termination sequencing (Sequenase, USB or Taq Track, Promega).

Expression/purification of endonuclease V

Expression of endonuclease V from ptac-denV and ptac-denV mutants was done as follows: an individual colony was grown overnight in NZY broth plus ampicillin and then used to inoculate 1 liter of broth with 100 μ g/ml ampicillin. After 3h, IPTG was added to a final concentration of 1 mM. After an additional 2h, cells were harvested by centrifugation at 5000 $\times$ g for 30 min. For induction experiments, 1 ml of cells was spun down and analyzed on 16% SDS-PAGE gels and stained with Fast Stain (Zion Research, Allston, ME). For purification of enzyme, cells were resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1mM β -ME, and 10% glycerol) and sonicated using a Branson sonicator, 5 $\times$ 20-sec pulses. The cellular debris was removed by centrifugation at 15,000 rpm/20 min in a JA-20 rotor at 4°C. The supernatant was then applied to a DEAE-cellulose column equilibrated in Buffer A plus 0.3M NaCl to remove nucleic acids. The unbound material was pooled and applied to a Blue-Sepharose column (Pharmacia) equilibrated with Buffer A plus 0.5 M NaCl. Endonuclease V was eluted in a 0.5–3.0 M NaCl gradient and the fractions from 2.0–2.5M were collected, pooled and concentrated. Protein purity was determined by SDS-polyacrylamide gel electrophoresis on 16% gels stained by a silver stain kit (Daichi/Enprotech, Hyde Park, MA). Protein concentration was determined by Bradford protein determination (Bio-Rad, Richmond, CA).

Analysis of endonuclease V mutants

In-vivo survival

In vivo survival of *uvr*⁻ host *E. coli* carrying the wild-type or mutant *denV* gene was evaluated as follows: A saturated overnight culture of ptac-denV/AB2480 (wild-type and mutant) grown in NZY broth with 100 μ g/ml ampicillin was diluted 1:100 in fresh broth and incubated with vigorous shaking for 3h. IPTG was added to 0.5 mM final concentration and cells were shaken for an additional hour. Cells were spun at 5000 $\times$ g for 5 min, washed 2 $\times$ in PBS to remove IPTG, resuspended in 10 ml of broth, and plated. After absorbtion, the plates were irradiated at 254 nm

Table II. Amino acid substitutions in denV mutants^a

	116	117	118	119	120	121
Wild-type	ALA	ARG	LEU	ASP	GLU	LYS
R117P		PRO				
L118F			PHE			
L118S			SER			
E120G					GLY	
K121I						ILE
K121Q ^b						GLN

^a Sequence based on Valerie et al., Reference 3.

^b From Altered-Sites Method (See Materials and Methods)

with a fixed UV lamp (Model XX-19) and the dose rate was monitored with a Black-Ray photometer (both from Ultraviolet Products, San Gabriel, CA). After irradiation, plates were incubated overnight in the dark to prevent photoreactivation. The D₃₇ value was based on that dose of UV that reduced survival to 37% of unirradiated controls.

Enzyme assays

Assays for total endonuclease activity, PD glycolylase, AP lyase activity and DNA binding were performed essentially as described (28,29). For DNA binding activity and AP lyase activity, [³H]-pBR322 irradiated at 300 and 200 J/m² respectively was used as substrate (for AP lyase activity, the substrate was depurinated in pH 4.5 buffer as described [30]). For PD glycosylase activity and total endonuclease activity, [³H]-poly(dA-dT) irradiated at 4200 J/m² was used as substrate. Assays were done in triplicate and calculations were based on the slope of the resulting curves as a percentage of wild-type activity.

RESULTS

In vitro mutagenesis of endonuclease V

A new strategy for developing a library of random mutants within a defined region was developed involving random targeted mutagenesis. The procedure involves direct PCR amplification of a mutated region and subsequent subcloning within an expression vector, in this case the over-expressing vector ptac-denV. This procedure requires no single-stranded template and can be performed directly from a double-stranded plasmid. In order to validate the procedure, we chose the Ala¹¹⁶ $\rightarrow$ Lys¹²¹ region of endonuclease V since the sequences coding for this region were centrally located within the cloning region of the expression vector, and as such should yield the highest number of mutants. Table II lists the mutants developed and used in this study.

In vivo survival of endonuclease V mutants

Survival curves were performed on 6 mutants of endonuclease V. Because over expression of endonuclease V is toxic to *E. coli*, it was important to control the induction of the enzyme in the host. We found that limited induction of 1h was sufficient for proper expression and that removing the inducer (IPTG) before plating/irradiation did not interfere with survival (data not shown). As can be seen in Fig. 3, most mutants had decreased survival when compared to wild-type enzyme. These mutants may be divided into two groups: those with wild-type survival ($D_{37} > 4.0$) such as L118F or L118S, and those with very low survival ($D_{37} < 2.0$) such as E120G. In no case was the resulting survival as low as that of the parent AB2480 control ($D_{37} \sim 0.4$) except the termination mutants such as L118STOP. This suggests that the 116–121 region is not as critical to enzyme function as the 128–132 region (see Discussion). As expected, even the wild-type enzyme could not restore full survival when compared to a *uvr*⁺ strain of *E. coli*. This is consistent with our previous observations on the capabilities of *denV* to restore survival. This may be due to several factors: the potential toxicity of over expression; the under expression of enzyme such that there is insufficient enzyme to repair the induced number of lesions; or the fact that Endonuclease V only repairs 5,6 cyclobutane dimers and not [6-4] photoproducts. It has been suggested that this latter lesion may in fact be the more important lesion with regard to survival (31).

Figure 3. Survival curves of Endonuclease V and Endonuclease V mutants (see Materials and Methods). Single mutations: ○, wild-type; ●, R117P; △, E120G; ▲, K121Q; □, K121I; ■, L118S; ▽, L118F; ◇, AB2480 (*uvr*⁻); ◆, X90 (*uvr*⁺).

Enzyme activities of endonuclease V mutants

Each of the 6 mutants of endonuclease V was analyzed for activities relating to substrate binding, pyrimidine dimer DNA-glycosylase, total endonuclease and AP lyase activity. The results of these assays are summarized in Table III. In general, for each activity, there were mutants that retained wild-type activity, mutants with intermediate activity, and mutants with no activity. Predicted neutral changes such as L118P or L118S had minimal effect on activity—L118S actually demonstrated increases in both DNA binding and PD-glycosylase activity. Amino acid substitutions expected to change tertiary structure radically indeed eliminated most activities: R117P and E120G had minimal survival and almost no enzymatic activity. Curiously, the E120G mutants, despite having no DNA binding or PD glycosylase activity, retained some AP lyase activity. These mutants, along with the K121I mutant—which has almost no AP lyase activity, while retaining binding, glycosylase and survival activity—lend credence to the idea that there is (i) no correlation between AP lyase activity and in vivo survival and (ii) no correlation between AP lyase activity and glycosylase activity. These data do demonstrate unequivocally the direct correlation between DNA binding or PD glycosylase activity and survival. Mutants such as K121Q have minimal AP lyase activity (4.1% of wild-type) yet retain wild-type levels of survival most likely due to increases in PD glycosylase activity (117%). This suggests that survival is primarily determined by first steps in initiating repair—the recognition and binding of the DNA substrate and the first cleavage of the glycosidic bond of the 5' thymine.

DISCUSSION

In this study, we have attempted to expand the structure/function map of the endonuclease V, a DNA repair enzyme from bacteriophage T4. To accomplish this, we have developed a fast and efficient method for generating a large library of mutants. The specificity of this enzyme is for the thymine dimer produced after UV irradiation. This enzyme has to perform four separate functions: recognition of the DNA substrate, binding to the DNA substrate, incision of the glycolytic bond through its pyrimidine-dimer DNA glycosylase activity, and cleavage of the phosphate backbone through its AP lyase activity. The location of the specific catalytic site(s) for these activities has been difficult to determine. The well documented mutants T4_{uvr13} (28, 32), which results in a termination codon at residue 128 (3), and T4_{F431} (33), which results in an Asp → Gly substitution at codon 133 (3), confirm the importance but not exclusive role

Table III. Summary of *in vivo* and *in vitro* activities of endonuclease V mutants

Mutant	Survival ^a	Binding ^b	PD glycosylase	AP endonuclease	Total endonuclease
Wild-type	4.6	100	100	100	100
R117P	1.9	2.4	3.3	4.2	5.3
E120G	1.9	0.5	3.4	35	0.8
K121Q	4.0	75	117	4.1	67
K121I	4.5	34	63	88	32
L118S	4.4	110	126	72	94
L118F	4.6	99	86	97	107
AB2480	0.4				

^a D_{37}

^b Based on the slope of the resulting curves relative to wild-type.

of the carboxy terminal region for catalytic activity. In addition, the mutant T4_{v1} (34)—the result of a -T deletion at codon 83 resulting in a premature termination codon at residue 118 (3)—has no activity (28). That this mutant has no activity is not surprising, since after codon 83 the amino acid sequence is nonsense through termination. Our mutant L118stop, on the other hand, is a true 'wild-type' termination mutant through codon 118 and has no activity in any of the assays measured (data not shown).

Other significant evidence for the role of the region from Tyr¹²⁸ → Tyr¹³² is found in the large number of aromatic residues known to be involved in at least the AP lyase activity. Laval and coworkers have demonstrated the capabilities of aromatic residues to break the phosphate backbone at AP sites (reviewed in 8). It has also been suggested that this region is important for both DNA binding and PD glycosylase activity (2). Mutagenesis studies in this region by both Lloyd and Ishida have demonstrated significant changes in both individual enzymatic activities and *in vivo* survival rates in most of their mutants. In fact most of the mutants in this region had profound effects on activity. Part of the problem in separating function and residue(s) is that this enzyme is very small (137 amino acids), and, as such, any change would be expected to produce significant changes in activity. In many cases, changes eliminated all activity. Again, the problem may be due simply to significant changes in three-dimensional conformation of the enzyme rather than any relationship of residue to function.

The more informative mutants, however, are those where partial activity is retained. Mutants such as Y129N/Y131N (10) and the E120G mutant presented here clearly demonstrate the independence of AP lyase activity from other activities and from survival. This may be explained by the notion that the AP lyase activity represents a 'scavenging' activity, secondary to the important glycosylase step. This is suggested by previous work on mutant endonuclease V such as that with K130H and K130G (9) and the T127M and K130L mutants of Ishida (11). This has now been confirmed by our work, demonstrating a clear relationship between binding and glycosylase activity and survival (Figure 3, Table III). The results presented here are consistent with the recent work using mutagenesis, reductive methylation and peptide analysis to demonstrate the integral role of the amino terminus in catalysis (12,13).

Thus, changes in the charged amino acids in the region we have chosen to study should result in changes reflecting these interactions. Mutations such as R117P and E120G eliminate activity. SDS-PAGE gels after induction rule out that these results are not due to the instability of the resulting protein (data not shown). In addition, mutants such as K121I also demonstrate the importance of these charged residues with respect to enzyme-substrate interaction.

Correlation of mutants with X-ray crystal structure

The X-ray crystallographic structure of Endonuclease V has been resolved to 1.6Å by Morikawa et al. (14). The structure shows a loop structure located at the carboxy terminal end containing the highly aromatic residue stretch Trp-Tyr-Lys-Tyr-Tyr (residues 128–132). Using the knowledge that basic residues can bind polynucleotides through electrostatic interactions, they were able to show that there is a significant accumulation of positive charges contributed by lysine and arginine residues that appear to lie along an internal curve of the molecule. Finally, analysis of the resolved structure focuses on the roles of Arg³

and Glu²³. Hydrogen bonding analysis reveals strong interaction of the side chain of Glu²³ which and the backbone imino group of Arg³. That Glu²³ is critical to enzyme function has been confirmed by new mutagenesis studies (15). Analysis of electrostatic potentials suggests that the Glu²³ is surrounded by several basic residues (e.g. Arg³, Arg²², Lys¹²¹) thus forming a region with a groove to accommodate substrate. This predicted importance of electrostatic binding of polynucleotides to basic residues is consistent with our observations and conclusions on the role of Arg¹¹⁷ and Lys¹²¹ in substrate binding and stabilization of the enzyme-substrate complex.

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ABBREVIATIONS

AP, apurinic/aprimidinic; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PD, pyrimidine dimer; T ⇄ T, thymine dimer; UV, ultraviolet.

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