Site-directed Mutagenesis of the NH₂ Terminus of T4 Endonuclease V

The Position of the αNH₂ Moiety Affects Catalytic Activity*

(Received for publication, June 24, 1992)

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Reductive methylation of the αNH₂ moiety of the DNA repair enzyme T4 endonuclease V has been shown previously to eradicate both the N-glycosylase and apyrimidinic/apurinic lyase activities of the enzyme (Schrock, R. D., III, and Lloyd, R. S. (1991) J. Biol. Chem. 266, 17631–17639). The present study uses the technique of site-directed mutagenesis to investigate the important parameters involved in the cleavage mechanism. The prediction was that the addition of an amino acid in the immediate NH₂-terminal region of the protein would alter the proximity of the αNH₂ moiety of Thr² to its target, thereby severely compromising the enzyme’s catalytic activity. However, substitutions in this region generally should be tolerated. To test this hypothesis, three substitutions of the NH₂-terminal amino acid were produced: Ser² (T2S), Val² (T2V), and Pro² (T2P). An addition mutant was also produced by adding a glycine between the first and second amino acids of the protein (Thr²-Gly-Arg³) (+Gly). The T2P and +Gly mutants had negligible pyrimidine dimer-specific nicking activity in vitro. Conversely, the T2S enzyme exhibited wild type levels of activity and the T2V exhibited intermediate levels of activity in vitro. Results from ultraviolet (UV) survival studies of the mutant enzymes indicated that the in vivo activities of these enzymes were directly correlated to the enzymes’ ability to cleave at pyrimidine dimers in vitro. These results indicate that a critical parameter for the functionality of endonuclease V is the relative distance between the primary αNH₂ group in the active site of the enzyme and those elements responsible for DNA binding and pyrimidine dimer recognition.

Ultraviolet (UV) light induces the formation of covalent photoproducts in DNA. The prevalent photoproduct is the cis-syn cyclobutane pyrimidine dimer (Patrick and Rahn, 1976; Cadet and Vigny, 1990). It is becoming increasingly clear that this particular photoproduct is mutagenic (Banerjee et al., 1988; Lawrence et al., 1990). The enzyme T4 endonuclease V initiates DNA repair at pyrimidine dimer sites. This 16-kDa gene product of the T4 denV gene has been purified to homogeneity, sequenced, and cloned in Escherichia coli (Radany et al., 1984; Valerie et al., 1984; Valerie et al., 1985; Recinos et al., 1986; Higgins and Lloyd, 1987; Lloyd and Augustine, 1986). This has allowed for extensive in vitro and in vivo analysis of its activities. The enzyme locates pyrimidine dimers using a salt-sensitive one-dimensional search along double-stranded DNA (Gruskin and Lloyd, 1986, 1988; Dowd and Lloyd 1989a, 1989b, Nickell et al., 1991). Endonuclease V then specifically binds to pyrimidine dimers (Radany and Friedberg; 1980) and cleaves the glycosylic bond of the 5'-pyrimidine of the pyrimidine dimer (Seawell et al., 1980; McMillan et al., 1981; Bonura et al., 1982). The phosphodiester bond 3' to the resulting abasic site of the pyrimidine dimer is then broken, producing an α,β-unsaturated aldehyde and a 5'-terminal phosphomonoester (Weiss and Grossman, 1987). Endonuclease V has also been shown to cleave the phosphodiester bond 3' to an abasic site by β-elimination (Manoharan et al., 1988; Kim and Linn, 1988). Although the two substrates of endonuclease V (abasic sites and pyrimidine dimers) are chemically and structurally different, it is likely that β-elimination of the phosphate through a Schiff base intermediate is the common mechanism for phosphodiester bond cleavage.

Chemical modification studies of endonuclease V utilizing the technique of reductive methylation demonstrated that the αNH₂ moiety of the enzyme is directly involved in the chemical mechanisms of both the glycosylase and apyrimidinic/apurinic lyase activities (Schrock and Lloyd, 1991). A reaction mechanism was proposed that incorporated these findings with previously determined physiochemical characteristics of the catalytic reactions of endonuclease V. Briefly, the mechanism involves a nucleophilic attack of the αNH₂ moiety at the C-1' of the deoxyribose of the 5'-pyrimidine of a dimer. The reactive covalent intermediate formed is a protonated Schiff base which can proceed through two pathways. The first pathway is a sequential β-elimination of the 3'-phosphate, resulting in cleavage of the phosphodiester bond. The products are an α,β-unsaturated aldehyde and a 5'-monophosphate. An alternative pathway leads to dissociation of the enzyme from the substrate, leaving an apyrimidinic/apurinic site. Reassociation of endonuclease V and subsequent cleavage of the phosphodiester bond at the abasic site is probable. The balance of the two pathways, dissociation after glycosyl bond scission or sequential phosphodiester bond cleavage, is affected by the concentration of nucleophiles in the solvent. For example, the enzyme dissociates after glycosyl bond cleavage more readily at pH 8.0 than at pH 6.8 (Nakabeppu and Sekiguchi, 1981). This increase in the rate of dissociation can be attributed to the increased concentration of OH⁻ at the higher pH. Furthermore, Liu et al.
parameters involved in the DNA cleavage mechanism of endonuclease V. We propose that a critical factor for the enzyme activity is the relative distance between the active site αNH₂ terminus and those domains responsible for DNA binding and pyrimidine dimer recognition. At the time of the study, complete crystallographic data on endonuclease V was not published. Our speculation that the NH₂ terminus is in a fixed position, poised in an ideal location to make the nucleophilic attack on the C-1° of the deoxyribose, has been since been found to be consistent with the published crystal structure of endonuclease V (Morikawa et al., 1992). We predicted that the addition or deletion of one amino acid in the immediate NH₂-terminal region of the protein would alter the proximity of the αNH₂ moity to its target, thereby severely compromising the enzyme's catalytic activity. However, a variety of substitutions of amino acids within this region should be tolerated. Earlier results from our laboratory obtained from site-directed mutagenesis of Arg residues supported this hypothesis. Briefly, this work involved the introduction of Glu, Asp, Glu, Lys and Arg all retained some aspect of catalytic activity, yet deletion of this residue resulted in a completely inactive enzyme (Dowd and Lloyd, 1989a, 1989b). At the time of those studies, it was errantly thought that the catalytic residue(s) resided within the COOH-terminal region of the enzyme.

The "proximity" hypothesis was tested by altering the enzyme at the genetic level using site-directed mutagenesis. Three substitutions of the NH₂-terminal amino acid, Thr², were produced: Ser² (T2S), Val² (T2V), and Pro² (T2P). Also, an addition mutant was produced by adding a glycine between the first and second amino acids of the protein (Thr²-Gly²-Arg² = +Gly). When combined with previous mutagenesis studies in the NH₂-terminal region of the protein, these results give further insight into the nature of the interaction of endonuclease V with its pyrimidine dimer substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—The origin of Escherichia coli strains, phage, and plasmids used in these studies is described in Table I. Polyvinylidene difluoride membrane was obtained from Bio-Rad. All other reagents were obtained from general suppliers.

**Oligonucleotide Site-directed Mutagenesis of denV**—The gene encoding endonuclease V, denV, and the transcriptional terminator sequences was previously constructed behind the hybrid A ÕP₄ hybrid promoter in an E. coli expression vector derived from bacteriophage M13 (Recinos and Lloyd, 1986; Recinos et al., 1986). Single-stranded uracil-containing M13mp18 DNA was prepared from bacteriophage M13-infected E. coli strain CJ236 (dut-ung +), and the DNA was diluted to 0.1 mg/ml in 25 mM NaH₂PO₄ buffer (pH 6.8), containing 10 mM EDTA (v/v) SDS, 15% (w/v) mercaptoethanol, and 0.02% (w/v) bromphenol blue and boiled for 5 min. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel and electroeluted onto a 0.2-μm polyvinylidene difluoride protein sequencing membrane at 0.4 mA for 1 h in polyvinylidene difluoride electroelution buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.9) in 10% (v/v) methanol) (Matsudaira, 1987). The membrane was stained for 5 min in 40% (v/v) methanol and 0.025% (w/v) Coomassie Brilliant Blue and destained 30 min with several changes of 50% (v/v) methanol solution at room temperature. The protein bands corresponding to wild type and mutant endonuclease V were excised from the membrane and used for Edman degradation microsequencing.

**Pyrimidine Dimer-specific Nicking Activity of Endonuclease V Mutants**—Form I pBR322 DNA was irradiated by 254-nm UV light at 100 μW/cm² for 245 s to generate 20–25 pyrimidine dimers/plasmid molecule (Gruskin and Lloyd, 1986). The DNA was diluted to 0.1 mg/ml in 25 mM NaH₂PO₄ buffer (pH 6.8), containing 10 mM EDTA, 20 mM NaCl, 10% (v/v) ethylene glycol, and 0.1 mg/ml bovine serum albumin. To 1 μg of pBR322 in the described buffer, varying concentrations of wild type or mutant endonuclease V were added. The DNA was incubated at 37°C for 5 min or a known amount of enzyme was added and incubated at 37°C for increasing amounts of time. The reaction was stopped by the addition of an equal volume of electrophoresis loading buffer (50 mM Tris-Cl buffer (pH 8.0) containing 50% (v/v) glycerol, 0.1% (w/v) mercaptoethanol, and 0.025% (w/v) bromphenol blue). The reaction products were subjected to electrophoresis through a 1% (w/v) agarose gel, stained with ethidium bromide (0.5 μg/ml) in electrophoresis buffer (40 mM sodium acetate buffer (pH 7.5) containing 10 mM EDTA, 1500 mM KCl, and 10% (v/v) ethylene glycol). Proteins in the eluent fractions were separated by 15% SDS-polyacrylamide gel electrophoresis and the fractions containing mutant enzyme identified by immunoblot analysis. The primary antibody used was a mouse monoclonal raised against a synthetic peptide containing an internal amino acid sequence of wild type endonuclease V (Prince et al., 1981). The region of recognition by the antibody was significantly distant from the NH₂-terminal region of the protein. The fractions containing mutant endonuclease V were pooled and NH₄SO₄ was slowly added to bring the final concentration to 1 M. The protein-containing solutions were loaded on a phenyl-Sepharose column equilibrated previously with buffer C (25 mM NaH₂PO₄ buffer (pH 6.8) containing 1 M (NH₄)₂SO₄, 100 mM KCl, and 10% (v/v) ethylene glycol). After a 1-column volume wash with buffer C, the mutant proteins were eluted with a 200-ml gradient of buffer C to buffer D (25 mM NaH₂PO₄ buffer (pH 6.8) containing 1 mM EDTA, 100 mM KCl, and 10% (v/v) ethylene glycol). Fractions containing mutant enzyme were determined as in the previous step by immunoblot analysis. Final purification in the purification scheme was to concentrate the enzyme and change the buffer to 50 mM NaH₂PO₄ buffer (pH 6.8) containing 1 mM EDTA in an Amicon system equipped with a YM10 filter under 50 p.s.i. of pressure. The final concentration of mutant endonuclease V proteins purified in this manner was determined by quantitative immunoblot analysis in which pure endonuclease V was used to generate a standard curve (Gruskin and Lloyd, 1988).

**Amino Acid Sequencing of Mutant Enzymes**—To prepare the purified wild type and mutant enzyme for amino acid sequencing, 40 μl of each enzyme were added to 75 μl of SDS loading buffer (200 μM sodium pyrophosphate containing 30% (v/v) glycerol, 15% (w/v) SDS, 15% (w/v) 2-mercaptoethanol, and 0.02% (w/v) bromphenol blue) and boiled for 5 min. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel and electroeluted onto a 0.2-μm polyvinylidene difluoride protein sequencing membrane at 0.4 mA for 1 h in polyvinylidene difluoride electroelution buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.9) in 10% (v/v) methanol) (Matsudaira, 1987). The membrane was stained for 5 min in 40% (v/v) methanol and 0.025% (w/v) Coomassie Brilliant Blue and destained 30 min with several changes of 50% (v/v) methanol solution at room temperature. The protein bands corresponding to wild type and mutant endonuclease V were excised from the membrane and used for Edman degradation microsequencing.
The advantage of using the uracil-containing M13mp18 O1p5denV template is that the original DNA template (wild type) is strongly selected against relative to the mutant template and thus the wild type strand is preferentially destroyed upon transfection (Kunkel et al., 1987). Also, the use of DNA mismatch repair-deficient E. coli host cells (NK7085) eliminates the correction of the intentionally mismatched bases. This combination of techniques resulted in >70% recovery of mutant denV genes. After the DNA sequence of each mutant was confirmed, the mutant genes were subcloned into the expression plasmid pCX2608 and transfected into repair-deficient E. coli.

**Purification of Mutant Endonuclease V**—Wild type and mutant forms of endonuclease V were expressed in the repair-deficient E. coli strain AB2480 utilizing the hybrid λ O1p5 promoter. The relative amounts of mutant enzymes were compared to wild type levels after electrophoresis of whole cell extracts in a 15% polyacrylamide SDS-containing gel. The proteins were electroblotted onto nitrocellulose and the wild type and mutant endonuclease V were detected by immunoblot analysis as described under "Experimental Procedures." It was found that the intracellular accumulation of each of the mutant enzymes was comparable to that of wild type (data not shown).

The mutant enzymes were purified as described under "Experimental Procedures." The concentration of each mutant enzyme was determined by quantitative immunoblot analysis and was typically on the order of 20–80 μg/ml. Each preparation was found to be free of nonspecific DNA nicking activity on supercoiled pBR322 DNA (data not shown).

**Amino Acid Sequencing of Mutant Enzymes**—In addition to confirming by DNA sequence analyses that the mutations had been created, it was also necessary to sequence the NH2-terminal amino acid residues of each mutant protein. Identical amounts of purified mutant and wild type endonuclease V proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to Edman degradation as described under "Experimental Procedures." The results are summarized in Table III. The relative yields obtained from each of the mutant proteins and the wild type enzyme were within 10% of each other, indicating that none of the mutant enzymes had blocked NH2 termini. As is observed for the wild type enzyme (Schrock and Lloyd, 1991), the NH2-terminal methionines of the T2S, T2V, and +Gly mutants are post-

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**Table 1**

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<th>Bacterial strain, plasmids, and phage used in this study</th>
<th>Genotype or phenotype</th>
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| CJ326 | dat1, ung1, thi1, relA1/pCJ105 CM' |
| NR7085 | Δ (lac pro 13) nald A mutS::Tn5 |
| UT481 | met thy Δ (lac-pro) hsdRBamHI hsd |
| AB2480 | wulA Δ M15 |
| pGX2608 | Δ A O1p5 λ t4 GalK' |
| pGX2608-16-denV* | Δ λ O1p5 endonuclease V' λ t4 GalK' |
| pGX2608-16-denV Thr2 → Pro2 | New 2 codon CCG |
| pGX2608-16-denV Thr2 → Ser2 | New 2 codon TCT |
| pGX2608-16-denV Thr2 → Val2 | New 2 codon GTT |
| pGX2608-16-denV Thr2, Arg2 → Thr2, Gly2+3 | Insertion of GGG between codons 2 and 3 |

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A. Ganesan, Stanford University
P. Modrich, Duke University
C. Joyce, Yale University
C. Lark, University of Utah
Genex Corp.
A. Ganesan, Stanford University
G. Recinos and Lloyd (1986)
A. Ganesan, Stanford University
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was as active as wild type enzyme, the T2V exhibited -40% activity was measured by loss of form I DNA. These measurements can be transformed on a semilog plot to give a first order rate constant. As shown in Fig. 2B, the T2S mutant showed negligible activity relative to wild type.

**Pyrimidine Dimer-specific Nicking Activity of Endonuclease V Mutants**—The pyrimidine dimer-specific nicking activities of the NH2-terminus mutants were evaluated in two separate assays. The first assay was an enzyme titration. Endonuclease V makes single-stranded nicks at pyrimidine dimer sites. Comparison of the pyrimidine dimer-specific nicking and pyrimidine dimer-specific glycosylase activities showed that the latter is slightly higher for the wild type enzyme, indicating that some dissociation of the enzyme before phosphodiester bond cleavage is an inherent property of endonuclease V activity (Nakabeppu and Sekiguchi, 1981). It was therefore necessary to determine whether the glycosylase activity of the NH2-terminal mutants was significantly higher than the pyrimidine dimer-specific nicking activity. The pyrimidine dimer-specific DNA glycosylase activity assay was identical to the nicking assay with the exception that the reaction products were treated with alkali to cleave the phosphodiester bond at residual abasic sites. Comparison of the pyrimidine dimer-specific nicking and pyrimidine dimer-specific glycosylase activities showed that the latter is slightly higher for the wild type enzyme, indicating that some dissociation of the enzyme before phosphodiester bond cleavage is an inherent property of endonuclease V activity (Nakabeppu and Sekiguchi, 1981). Fig. 3 shows the pyrimidine dimer-specific DNA glycosylase activities of the wild type and mutant forms of endonuclease V relative to the pyrimidine dimer-specific nicking activity of each enzyme. None of the mutant enzymes showed a significantly higher rate of glycosylase activity over pyrimidine dimer-specific nicking activity. Thus, none of the mutations resulted in an enzyme that has a higher rate of dissociation before cleavage of the glycosylolytic bond and before cleavage of the phosphodiester bond.

**Pyrimidine Dimer-specific DNA Glycosylase Activity of Endonuclease V Mutants**—The catalytic mechanism of endonuclease V allows for the dissociation of the enzyme from the pyrimidine dimer substrate after cleavage of the glycosylolytic bond but before cleavage of the phosphodiester bond under some circumstances (Schrock and Lloyd, 1991). Certain mutations of the denV gene have affected glycosylase activity and phosphodiester lyase activity differentially (Seawell et al., 1980; Valerie et al., 1984, Stump and Lloyd, 1988; Recinos and Lloyd, 1988). It was therefore necessary to determine whether the glycosylase activity of the NH2-terminal mutants was significantly higher than the pyrimidine dimer-specific nicking activity. The pyrimidine dimer-specific DNA glycosylase activity assay was identical to the nicking assay with the exception that the reaction products were treated with alkali to cleave the phosphodiester bond at residual abasic sites. Comparison of the pyrimidine dimer-specific nicking and pyrimidine dimer-specific glycosylase activities showed that the latter is slightly higher for the wild type enzyme, indicating that some dissociation of the enzyme before phosphodiester bond cleavage is an inherent property of endonuclease V activity (Nakabeppu and Sekiguchi, 1981). Fig. 3 shows the pyrimidine dimer-specific DNA glycosylase activities of the wild type and mutant forms of endonuclease V relative to the pyrimidine dimer-specific nicking activity of each enzyme. None of the mutant enzymes showed a significantly higher rate of glycosylase activity over pyrimidine dimer-specific nicking activity. Thus, none of the mutations resulted in an enzyme that has a higher rate of dissociation before cleavage of the glycosylolytic bond and before cleavage of the phosphodiester bond.
Results from the reductive methylation of the NH2 terminus of endonuclease V indicated that the αNH2 moiety is directly involved in the chemical mechanism of cleavage of both pyrimidine dimer-containing and abasic site-containing substrates (Schrock and Lloyd, 1991). To further explore the catalytic site of endonuclease V, site-directed mutagenesis of the NH2-terminal region was performed. It was postulated that the αNH2 moiety was in a fixed location, poised for nucleophilic attack on the C-1’ of the deoxyribose moiety. Extending or deleting the NH2-terminal part of the protein would therefore compromise the enzyme’s ability to cleave at cyclobutane pyrimidine dimers. It follows that a variety of substitutions would be tolerated at the NH2 terminus without the loss of catalytic activity as long as the αNH2 moiety of the mutant remained unblocked.

Four mutants of endonuclease V were produced, differing in the immediate NH2-terminal region of the protein. Three substitutions of the first amino acid of the protein were made, the most conserved being T2S and T2V. The substitution of proline for Thr3 (T2P) was designed to probe whether the steric restraints of the pyrrolidine ring would be detrimental to the enzyme’s viability. The glycine addition between Thr2 and Arg3 (+Gly) was expected to result in an inactive enzyme.

An important consideration in the production of the NH2-terminal mutants was the effect of these mutations on post-translational modification of the protein. The nature of post-translational modification within the cell is determined largely by the second amino acid (after formylmethionine) of the protein (Flinta et al., 1986). Although the NH2-terminal formylmethionine of the wild type enzyme is post-translationally cleaved in E. coli (Schrock and Lloyd, 1991), it was entirely possible that such modifications might not occur in the cells expressing the mutant enzymes. The results of the amino acid sequencing reactions (Table III) indicated that the NH2-terminal methionines of the T2S, T2P, and +Gly mutants were post-translationally cleaved within the cell. However, the T2V mutant preparation proved to contain a mixture; 60% of the mutant beginning with methionine and 40% beginning with valine. This meant that, in effect, 60% of the T2V enzyme preparation was actually an “addition” mutant, whereas only 40% was a substitution mutant.

The in vitro pyrimidine dimer-specific nicking activity of the four mutant enzymes was compared to wild type endonuclease V in two assays. The first assay demonstrated the nicking activity of the enzymes on UV-irradiated plasmid DNA using increasing concentrations of enzyme at a given time point (Fig. 1). The second assay demonstrated the time-dependent nicking activity of each enzyme at a constant enzyme concentration (Fig. 2). In both assays, the T2P and +Gly mutants demonstrated very little pyrimidine dimer-dependent nicking activity, the T2V mutant demonstrated intermediate activity, and the T2S mutant was identical to that of the wild type enzyme.

In addition, the pyrimidine dimer-specific N-glycosylase activity of each mutant enzyme was assessed. If the mutant enzyme was able to successfully cleave the glycosylic bond and dissociate from the pyrimidine dimer without concomitant cleavage of the phosphodiester bond, the rate of formation of alkali-labile abasic activity would be much higher than the rate of pyrimidine dimer-specific nicking. As shown in Fig. 3, none of the mutant enzymes possessed an unusually high rate of N-glycosylase activity over the incision of the phosphodiester bond at pyrimidine dimer sites.

The results of the in vitro activity assays support the predictions made about the effects that these mutations would have on endonuclease V. The T2S mutant behaved exactly like wild type, whereas the T2P and +Gly mutants were relatively inactive. The best explanation for the decreased activity of the T2V mutant stems from the fact that there are two different populations of enzyme present in the preparation. As previously mentioned, 60% of the T2V mutant is actually an addition mutant, much like +Gly. The deformed methionine on the NH2 terminus of 60% of the protein molecules likely deems this portion of the enzyme population...
FIG. 3. Pyrimidine dimer-specific glycosylase activity of endonuclease V mutants: time course assay. Wild type (0.03 μg/ml) or mutant endonuclease V (0.03 μg/ml) was added to UV-irradiated form I pBR322 in duplicate reactions. After incubation at 37°C for 0–30 min, NaOH was added to 100 mM to one of each of the duplicates. The remaining percent of form I DNA was determined for both + and − NaOH by agarose gel electrophoresis. For all panels, the following symbols are used: □, wild type; −NaOH; ■, wild type; +NaOH; ○, mutant; −NaOH; ●, mutant; +NaOH. Each panel represents data from a different mutant: A, T2S; B, +Gly; C, T2V; D, T2P.

FIG. 4. In vivo assay of mutant enzyme activity. The in vivo activity of the mutant enzymes was determined by UV survival experiments. The enhanced UV resistance of repair-deficient E. coli harboring the mutant denV constructs were compared with cells containing the wild type gene or only the parent vector. The percentage of surviving cells was determined by dividing number of colonies which grew after UV irradiation by the number of colonies grown on the unirradiated plates. □, wild type (pGX2608-denV'); ○, T2S; □, T2V; ●, T2P; ■, +Gly; ●, pGX2608.

inactive. However, 40% of the enzyme population is of the “correct” length. This is presumed to be the portion of the population which is catalytically active. Therefore the concentration of active enzyme is 40% of the total concentration of enzyme.

Studies of the in vivo activity of the mutant enzymes exactly correlate to the in vitro studies. The intracellular accumulation of the mutant enzymes was determined to be within 10% that of wild type. As shown in Fig. 4, the T2P and +Gly mutations did not enhance the the UV resistance of repair-deficient E. coli. The T2V mutant exhibited approximately 40% the enhancement of UV resistance that wild type enzyme endows to the cells, whereas the T2S mutant enhanced UV resistance to the level found in cells harboring the wild type denV gene. As in the in vitro studies, the decreased activity of the T2V mutant most likely results from the lower in situ concentration of active enzyme. It is quite clear that substitution of Thr with serine or valine does not compromise the catalytic activity of endonuclease V in vitro or in vivo. However, it is apparent from the incomplete post-translational cleavage of Met from the T2V mutant (resulting in a partially active population of enzyme) that such a post-translational event is important for the production of active enzyme in the cell.

The requirement that the αNH₂ moiety be in a certain position with respect to domains of the protein that are responsible for pyrimidine dimer-specific binding seems to be stringent. First, the addition of one amino acid (+Gly) is enough to inactivate the enzyme. Furthermore, as demonstrated by the inactivity of the T2P mutant, the positioning of the αNH₂ group may even be sensitive to the small change in placement of the secondary amino group imposed by the pyrrolidine ring of proline. A consequence of this change in position is that the secondary amino group of the proline would not in position to act as a nucleophile on the substrate. Alternatively, the pyrrolidine ring may itself sterically keep the amino group from accessing the C-1' of the deoxyribose moiety of the pyrimidine dimer. The cause of the loss of activity is likely to be steric rather than chemical, as the secondary amine of the NH₂-terminal proline should still act as a nucleophile. Our postulation was that the NH₂-terminal region lies in an immobile region of the enzyme, poised in a perfect position to act as a nucleophile with the C-1' moiety of the pyrimidine dimer substate. However, the αNH₂ moiety itself needs to be accessible enough to get within a reasonable distance of the electrophile. Thus, any other type (i.e. secondary amine) of amino group would not be sufficiently accessible.

After the completion of this study, the x-ray crystal structure of endonuclease was published by Morikawa et al. (1992).
They showed that the seven amino acids at the NH₂ terminus protrude between two major alpha helices and that Thr² and Arg² lie on the molecular surface of a putative DNA binding surface. In addition, the side chain of Glu²³ forms hydrogen bonds with the Arg² side chain and with the peptide backbone carbonyl group. Morikawa et al. (1992) state that Glu²³ lies in the catalytic center. While formation of a Schiff base intermediate between the Glu²³ side chain and the DNA substrate is chemically impossible, Glu²³ may have a distinct role in the chemical mechanism of cleavage. As suggested by Schrock and Lloyd (1991), protonation of the 5'-pyrimidine ring associated with the pyrimidine dimer could facilitate cleavage and Lloyd (1991), hydrogen bonds between the NH₂-terminal amino acids and Thr² and Arg² lie on the molecular surface of a putative DNA binding surface. In addition, the side chain of Glu²³ forms hydrogen bonds with the Gua² side chain and with the DNA substrate.

In conclusion, the results from this study strongly support the hypothesis that the proximity of the αNH₂ moiety of the catalytic site of the enzyme to other important structural features within the enzyme is a critical parameter for the viability of T4 endonuclease V. These results, when taken in context with the crystal structure of endonuclease V, make the active site of this enzyme the most understood of any N-glycosylase.

**REFERENCES**


