Consequences of Molecular Engineering Enhanced DNA Binding in a DNA Repair Enzyme†

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ABSTRACT: Facilitated one-dimensional diffusion is a general mechanism utilized by several DNA-interactive proteins as they search for their target sites within large domains of nontarget DNA. T4 endonuclease V is a protein which scans DNA in a nonspecifically bound state and processively incises DNA at ultraviolet (UV)-induced pyrimidine dimer sites. An electrostatic contribution to this mechanism of target location has been established. Previous studies indicate that a decrease in the affinity of endonuclease V for nontarget DNA results in a decreased ability to scan DNA and a concomitant decrease in the ability to enhance UV survival in repair-deficient *Escherichia coli*. This study was designed to question the contrasting effect of an increase in the affinity of endonuclease V for nontarget DNA. With this as a goal, a gradient of increasingly basic amino acid content was created along a proposed endonuclease V–nontarget DNA interface. This incremental increase in positive charge correlated with the stepwise enhancement of nontarget DNA binding, yet inversely correlated with enhanced UV survival in repair-deficient *E. coli*. Further analysis suggests that the observed reduction in UV survival is consistent with the hypothesis that enhanced nontarget DNA affinity results in reduced pyrimidine dimer-specific recognition and/or binding. The net effect is a reduction in the efficiency of pyrimidine dimer incision.

The rates of biological reactions are often limited by the rates at which diffusion can bring together the reactants. Thus, the rates of crucial DNA-interactive reactions are restricted by the efficiency with which DNA-interactive proteins locate their target sequences or structural aberrations among a vast excess of nontarget DNA. Facilitated one-dimensional diffusion along nontarget DNA is a mechanism of target site location utilized by several DNA-interactive proteins. As these proteins scan or slide along DNA in a nonspecifically bound state, the dimensionality of the target search is limited. The efficiency of the resulting target search is greater than that expected for a simple three-dimensional diffusion-controlled process [for reviews, see Lohman (1986), Ptashne (1986), Mazur and Record (1989), and von Hippel and Berg (1989)]. Scanning has been observed for *Escherichia coli* lac repressor (Riggs et al., 1970; Barkley, 1981; Berg et al., 1981, 1982; Winter & von Hippel, 1981; Winter et al., 1981), *Eco*RI endonuclease (Jack et al., 1982; Langowski et al., 1983; Ehbrecht et al., 1985; Terry et al., 1985), RNA polymerase (Belinstev et al., 1980; Hannon et al., 1980; Park et al., 1982; Roe & Record, 1985; Singer & Wu, 1987, 1988; Wheeler et al., 1987), bacteriophage λ cro protein (Kim et al., 1987), *Bam*HI endonuclease, *Bam*HI methylase (Nardone et al., 1986), and *Micrococcus luteus* UV endonuclease (Hamilton & Lloyd, 1989).

In addition to the above-mentioned proteins, T4 endonuclease V has also been observed to one-dimensionally scan nontarget DNA (Lloyd et al., 1980; Ganesan et al., 1986; Gruskin & Lloyd, 1986). Endonuclease V initiates the repair of ultraviolet (UV)[1]-induced cyclobutane pyrimidine dimers in T4-infected *E. coli* [reviewed by Dodson and Lloyd (1989)]. Initially, the enzyme binds nonspecifically to DNA and slides on the nontarget DNA until it encounters and binds to a pyrimidine dimer. The mechanism of incision consists of the sequential action of a DNA glycosylase which cleaves the glycosyl bond of the 5′-pyrimidine of the dimer and an apyrimidinic lyase activity which cleaves the phosphodiester bond between the two pyrimidines via a β-elimination reaction (Gordon & Haseltine, 1980; Haseltine et al., 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; Schrock & Lloyd, 1991). In in vitro assays at monovalent salt concentrations less than 40 mM, endonuclease V slides on nontarget DNA sequences, generating incisions in DNA at the sites of pyrimidine dimers by a processive nicking mechanism. At monovalent salt concentrations above 40 mM, the enzyme no longer scans nontarget DNA sequences in a linear search for pyrimidine dimers. Rather, the target location mechanism becomes a random three-dimensional search which results in a distributive nicking mechanism. Although the *E. coli* intracellular cation concentration is considerably greater than 40 mM, in vivo studies demonstrate that endonuclease V acts processively under normal *E. coli* physiological conditions and that the processive incision of pyrimidine dimers is of great biological significance (Gruskin & Lloyd, 1988; Dowd & Lloyd, 1989a,b, 1990). Endonuclease V has also been shown to interact with DNA in vitro as well as in vivo as a protein homodimer during the pyrimidine dimer search (Nickell & Lloyd, 1991).

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[1] Abbreviations: UV, ultraviolet; LB, Luria broth; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; AP, apurinic/apyrimidinic.

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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		
CJ236	dut1,ung1,thi1,relA1/pCJ105(Cm^r)	C. Joyce, Yale University
NK7085	Δ(lac pro 13) nal A mutS::Tn5	P. Modrich, Duke University
UT481	met thy Δ(lac-pro) hsdRBamHI hsdM$^+$ supDTn10/F′ traD36 proAB lacIqZΔM15	C. Lark, University of Utah
AB2480	uvrA6 recA13	A. Ganesan, Stanford
phage		
M13mp18-O_LP_R-*den*V		Recinos & Lloyd (1986)
M13mp18-O_LP_R-*den*V-A30K,V31L		this study
M13mp18-O_LP_R-*den*V-A30K,V31L,N37K		this study
M13mp18-O_LP_R-*den*V-A30K,V31L,H34K,N37K		this study
plasmid		
pGX2608	Ap^{r}λO_LP_Rλt_{4s} GalK$^+$	Genex Corp.
pGX2608-16-*den*V$^+$	Ap^{r}λO_LP_R endonuclease V$^+$ λt_{4s} GalK$^+$	Recinos & Lloyd (1986)
pGX2608-*den*V-A30K,V31L	new 30 and 31 codon	this study
pGX2608-*den*V-A30K,V31L,N37K	new 30, 31, and 37 codon	this study
pGX2608-*den*V-A30K,V31L,H34K,N37K	new 30, 31, 34, and 37 codon	this study

Previous computer-assisted modeling and site-directed mutational studies suggest that an endonuclease V–nontarget DNA interface exists along the highly basic, solvent-exposed face of a putative endonuclease V α-helix, including at a minimum amino acid residues 26–37 (Nickell et al., 1991; Dowd & Lloyd, 1990; Augustine et al., 1991). Furthermore, the manipulation of electrostatic character in this region indicates the presence of key sites of endonuclease V–nontarget DNA interaction. In these studies, the abolition of positive charges at amino acid residues Arg-26 and Lys-33 resulted in a decreased electrostatic attraction for DNA, an increased protein–DNA dissociation rate, and a subsequent decrease in the ability of these mutants to scan DNA (Dowd & Lloyd, 1990). In contrast, the addition of single-point positive charges at amino acid positions Ala-30, His-34, or Asn-37, along the proposed solvent-exposed α-helical face, resulted in an enhanced endonuclease V nontarget DNA binding affinity, yet did not alter the biological activities of these enzymes relative to native endonuclease V (Augustine et al., 1991; Nickell et al., 1991). To further address the biological effect of an enhanced nontarget DNA affinity, we chose to use simultaneous, site-directed mutations at amino acid positions Ala-30, His-34, and Asn-37, in order to create a gradient of increased positive charge along the proposed endonuclease V–nontarget DNA interface.

Materials and Methods

Materials. All chemicals were reagent grade. All solutions were prepared with distilled, deionized (Milli-Q) water. Mutagenic oligonucleotides were obtained from Research Genetics, Huntsville, AL. [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from New England Nuclear. [^{3}H]Thymidine was obtained from Amersham. Nitrocellulose membrane filters were purchased from Sartorius. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were obtained from New England Biolabs. NA45 DEAE DNA binding and recovery membranes were purchased from Schleicher & Schuell. [^{3}H]pBR322 was isolated by the alkaline lysis/CsCl–ethidium bromide equilibrium centrifugation method (Radloff et al., 1967; Birnboim, 1983). The *E. coli* strains, M13 phage constructs, and plasmids used in this study are described in Table I.

Oligonucleotide Site-Directed Mutagenesis of denV. The structural gene encoding endonuclease V, *den*V, and transcription termination sequences have been previously reconstructed behind the hybrid λ O_LP_R promoter in the *E. coli* expression vector M13 bacteriophage (Recinos & Lloyd, 1986; Recinos et al., 1986). Mutagenesis of the *den*V gene was performed as described by Nickell et al. (1991). The sequences of the mutagenic oligonucleotides were designed from the published *den*V sequence (Radany et al., 1984; Valerie et al., 1984). An initial mutagenic oligonucleotide was designed to encode the change from Ala-30, Val-31 (GCAGTT) to Lys-30, Leu-31 (AAACTT) (a 21-mer with sequence 5′-CTTACGAAGTTTACCAAAAAC-3′). The accompanying mutation, V31L, was also incorporated into the gradient of increased positively charge mutants described below so that no bias would be introduced during the comparison of the present increased positively charge mutants with published data regarding the A30K,V31L mutant protein. Following the generation and DNA sequence confirmation (Sanger et al., 1977) of this mutation, a second mutation was added to the *den*V sequence using the A30K,V31L mutant *den*V sequence as a template. This second mutation was designed using a mutagenic oligonucleotide intended to alter Asn-37 (AAC) to Lys-37 (AAA) (a 21-mer with sequence 5′-ACGTTTACCTTTAGCAACATG-3′). Following the generation and DNA sequence confirmation of this second mutation, a third and final mutation was added to the *den*V sequence using the A30K,V31L,N37K mutant *den*V sequence as a template. This final mutation was generated using a mutagenic oligonucleotide designed to encode the change from His-34 (CAT) to Lys-34 (AAA) (a 21-mer with sequence 5′-TTTAGCAACTTTCTTACGAAG-3′). Following the sequence verification of this final mutation, double-stranded mutant replicative form (RF) M13 DNA from the A30K,V31L,N37K and A30K,V31L,H34K,N37K mutants was prepared (Zoller & Smith, 1983), and the mutant *den*V gene inserts were released by *Cla*I restriction digestion. The mutant *den*V gene inserts were isolated using S&S NA45 DEAE membranes and subcloned into the *E. coli* expression vector pGX2608 (Recinos & Lloyd, 1986) at the unique *Cla*I site. These plasmids were transformed into *E. coli* UT481. Following ampicillin selection, the desired insert orientation was confirmed by diagnostic restriction analysis (Recinos & Lloyd, 1986). Plasmids containing the mutant *den*V genes pGX2608-*den*V-A30K,V31L,N37K and pGX2608-*den*V-A30K,V31L,H34K,N37K in the proper orientation were then transformed into *E. coli* AB2480 (uvrA$^-$ recA$^-$).

Preparation of Mutant Enzymes. Following the construction of the mutant *den*V-containing plasmids, the mutant endonuclease V proteins were expressed using the λ O_LP_R hybrid promoter in *E. coli* AB2480 grown at 30 °C for 16 h in LB media supplemented with 100 μg/mL ampicillin. Cells (2 L) were pelleted by centrifugation at 4500*g* and resuspended in 100 mL of cold 20 mM Tris-HCl (pH 8.0), 10 mM EDTA,

200 mM KCl, and 10% (v/v) ethylene glycol. Cells were disrupted by sonication and the cell debris removed by centrifugation at 10000*g*. Wild-type endonuclease V and the two mutant proteins were purified by the sequential use of the following chromatography steps: single-stranded DNA agarose, G-100 gel filtration, and heparin–Sepharose (Prince et al., 1991). Following this purification scheme, all enzyme preparations were found to be free of nonspecific DNA nicking activity. The amount of the mutant endonuclease V proteins recovered by this method was determined by quantitative Western blot analyses in which pure endonuclease V was used to generate a standard curve (Gruskin & Lloyd, 1988).

Pyrimidine Dimer-Specific Nicking Activity. Form I [^{3}H]pBR322 was irradiated by 254-nm UV light at 100 μW/cm^2 for 330 s in order to generate ~10 pyrimidine dimers per plasmid molecule (Gruskin & Lloyd, 1986). The DNA was then diluted to 0.05 mg/mL in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 10–110 mM KCl as described in the figure legends. To 1 μg of [^{3}H]pBR322 in the described solution were added varying concentrations of wild type or mutant endonuclease V, and the mixture was incubated at 37 °C for the time period described in the figure legends. Alternatively, a fixed concentration 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% sucrose, 2% SDS, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.01% bromophenol blue]. The reaction products were subjected to electrophoresis through a 1% (w/v) agarose gel, stained with ethidium bromide in electrophoresis buffer which consisted of 40 mM sodium acetate and 2 mM EDTA (pH 8.0), and the topological forms of DNA were visualized by exposure to long-wave UV light. DNA forms I, II, and III were excised and placed in scintillation vials with 150 μL of 1 N HCl. After the agarose was melted, 10 mL of aqueous scintillation fluid was added to each vial, and the radioactivity was determined by liquid scintillation spectroscopy (Lloyd et al., 1980).

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

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

Combined Pyrimidine Dimer- and AP-Specific Nicking Activity. Acid-depurinated [^{3}H]pBR322 DNA was prepared as described above. Following ethanol precipitation, the pellet was solubilized in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM KCl to a concentration of 0.5 mg of DNA/solution. This solution was then irradiated by 254-nm UV light at 100 μW/cm^2 for 330 s in order to generate ~10 pyrimidine dimers per plasmid molecule (Gruskin & Lloyd, 1986). The acid-depurinated, pyrimidine dimer-containing DNA was then diluted to 0.05 mg/mL in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/mL BSA, and 10 mM KCl. To 1 μg of [^{3}H]-pBR322 in the described solution were added varying concentrations of wild type or mutant endonuclease V, and the mixture was incubated at 37 °C for 30 min. The reaction was terminated and its products were analyzed as described for pyrimidine dimer-specific nicking activity.

Results

Design, Construction, and Production of Mutant Endonuclease V Protein in Cells. In previous studies, an endonuclease V–nontarget DNA interface has been shown to involve the amino acid residues at positions Arg-26, Ala-30, Lys-33, His-34, and Asn-37, all of which lie along a proposed solvent-exposed face of a putative α-helix (Dowd & Lloyd, 1990; Augustine et al., 1991; Nickell et al., 1991). The single substitution of lysine at positions Ala-30, His-34, or Asn-37 resulted in an enhanced nontarget DNA binding activity. To further address these observations, we constructed additional mutant *den*V genes in order to create a series of altered proteins which represent a gradient of increasing positive charge along this α-helix. Using oligonucleotide site-directed mutagenesis, the endonuclease V mutant enzymes containing the A30K,V31L,N37K and A30K,V31L,H34K,N37K alterations were created. The wild-type enzyme and the mutants of T4 endonuclease V were expressed utilizing the hybrid λ O_LP_R promoter in repair-deficient *E. coli* AB2480 cells. The steady-state intracellular levels of wild-type endonuclease V and each of the mutants were compared by Western blot analysis following SDS–polyacrylamide gel electrophoresis of total protein in whole cell extracts. It was observed that each of the mutant proteins accumulated intracellularly at levels 80–100% of the wild-type enzyme (data not shown). Following the purification of the enzymes as described under Materials and Methods, the concentrations of the partially purified mutant enzymes were quantitated by Western blot analysis (Gruskin & Lloyd, 1988). All preparations were found to be free of nonspecific DNA nicking activity (data not shown).

T4 Endonuclease V Dimer-Specific Nicking Activity in the Presence of Competitor DNA—An Assay for Relative Nontarget DNA Affinity. The electrostatic character of the protein–nontarget DNA interaction is known to contribute to the nontarget DNA scanning ability of endonuclease V. These interactions are sensitive to changes in in vitro ionic strength. At low salt concentrations, the basic amino acids are more tightly associated with the negatively charged phosphate groups along the DNA backbone. This leads to the decreased dissociation rate associated with sliding. As the salt concentration of the reaction environment increases, the attraction of the protein for nontarget DNA is diminished. As a consequence, the protein dissociates from the DNA at a higher rate, and the target location mechanism becomes distributive (Leirmo et al., 1987). This feature is demonstrated by the monovalent ionic strength-dependent competition of unirradiated DNA with pyrimidine dimer-containing DNA (Gruskin & Lloyd, 1986; Augustine et al., 1991; Nickell et al., 1991). A series

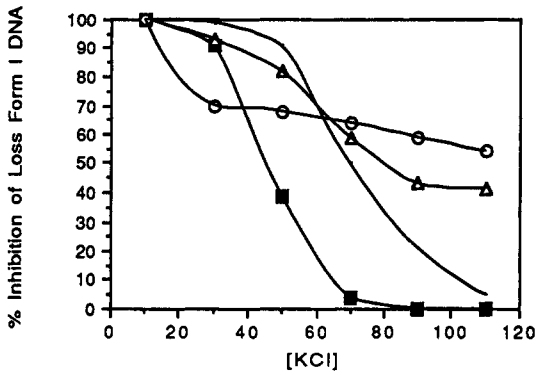


FIGURE 1: Percent inhibition of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers in the presence of unirradiated calf thymus DNA. Partially purified endonuclease V (1.0 ng) was added to 1.0 μg of UV-irradiated [^{3}H]pBR322 in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, various concentrations of KCl, and 1 mg/mL BSA either in the presence or in the absence of a 10-fold excess of unirradiated calf thymus DNA. Solutions were incubated for 60 min at 37 °C. Following the termination of the reaction, the three topological forms of DNA were separated on a 1% agarose gel, and the fraction of form I remaining in the presence or absence of the excess competitor DNA was determined by scintillation spectroscopy. The percent inhibition of form I DNA loss in the presence of competitor calf thymus DNA was determined by a comparison to control reactions containing no calf thymus DNA. Endonuclease V (wild type) (■); A30K,V31L,N37K (△); A30K,V31L,H34K,N37K (○). The previously published percent inhibition curve for the A30K,V31L mutant is shown as a solid line. This curve has been normalized to the current experimental level of percent form I DNA remaining.

of pyrimidine dimer-specific nicking assays were performed at increasing KCl concentrations, both in the absence an in the presence of a 10-fold excess of unirradiated calf thymus DNA, in order to determine if the alterations in the electrostatic characteristics of the modified endonuclease V molecules affected nontarget DNA interaction. This assay measured the salt concentration at which a processive endonuclease V reaction becomes distributive. The percent inhibition of form I DNA loss in the presence of competitor calf thymus DNA was then determined by a comparison to control reactions containing no calf thymus DNA (Figure 1). Included on this figure are the previously published inhibition values of the native enzyme and a mutant enzyme which contained one additional basic residue, A30K,V31L (Nickell et al., 1991).

At KCl concentrations where processivity is high, the loss of pyrimidine dimer-containing form I plasmid DNA is inhibited by excess unirradiated DNA. Under distributive KCl concentrations, the presence of up to a 25-fold excess of competitor DNA does not affect the random target search mechanism. The salt concentration at which the wild type endonuclease V target site location mechanism switches from processive to distributive is approximately 40 mM (Gruskin & Lloyd, 1986; Nickell et al., 1991). As described earlier, upon increasing the net positive charge on endonuclease V by +1 at amino acid residue 30, the A30K,V31L enzyme showed enhanced nontarget DNA binding affinity. This enhancement was demonstrated by a shift in the percent inhibition curve to higher KCl concentrations, with an increase in the point of mechanistic transition for target search from ~40 to ~80 mM. Further increase in the net positive charge of endonuclease V, as demonstrated by the A30K,V31L,N37K mutant inhibition curve, resulted in a further increase in the KCl concentration over which endonuclease V exhibits increased nontarget DNA binding. The effect of this enhanced nontarget DNA affinity was manifested even at the highest experimental salt concentration. An additional sequential increase in the net positive charge on endonuclease V to +3, with the

Table II: Effect of Ionic Strength on the Initial Pyrimidine Dimer Incision Rate

endonuclease V	net charge increase	rate at 100 mM KCl/rate at 10 mM KCl[a]
wild type	0	24
A30K,V31L	+	13.8
A30K,V31L,N37K	++	6.1
A30K,V31L,H34K,N37K	+++	4.1

[a] Rate = $[(-\ln \text{form I DNA}_{t_2}) - (-\ln \text{form I DNA}_{t_1})]/(\text{time}_{t_2} - \text{time}_{t_1})$.

A30K,V3L,H34K,N37K mutations, resulted in a percent inhibition curve whose shape was different from those of enzymes which are capable of making the transition from a processive to a distributive target search mechanism. In the case of this latter mutant, there was no point of mechanistic transition as indicated by the absence of a KCl concentration at which the presence of competitor DNA can result in a 50% inhibition of pyrimidine dimer-containing form I DNA nicking. The presence of unirradiated competitor DNA had a significant inhibitory effect at all the shown KCl concentrations. Thus, these data demonstrate that the incremental increase in the net positive charge of these endonuclease V mutants established a gradient of enhanced nontarget DNA affinity. However, these data demonstrate that there is not a one-to-one correspondence between enhanced positive charge and nontarget DNA binding affinity. Furthermore, the altered competition curves suggest that the generation of this gradient of increased positive charge results in additional modifications in endonuclease V character.

Differential Mechanisms by Which Altered Endonuclease V Molecules Locate Pyrimidine Dimers Are Affected by Salt Concentration. In a pyrimidine dimer-specific nicking reaction, the cleavage of supercoiled form I plasmid DNA generates form II (nicked circular) or form III (linear) plasmid molecules. Double-strand DNA breaks are produced when two incisions occur in close proximity on opposite DNA strands. For a processive nicking mechanism, all pyrimidine dimers located on a plasmid are incised prior to the dissociation of the enzyme from the DNA molecule, resulting in a linear accumulation of form III plasmid DNA over time. At monovalent salt concentrations above 40 mM, the target location mechanism is three-dimensional and, thus, pyrimidine dimer-specific nicking becomes randomly distributed among the plasmid DNA molecules. The observed rate of form I DNA loss increases while the accumulation of form III DNA is delayed until a sufficient number of random breaks have been introduced, such that there is a high probability that breaks in close proximity on opposite DNA strands will be made.

To characterize the effect of salt concentration on the mechanism of target site location, a kinetic analysis was performed under conditions in which the wild-type enzyme exhibits processive and distributive search mechanisms (Figures 2 and 3). When the fractions of form I DNA remaining (Figures 2 and 3, panel A) were transformed to first-order rate constants (panel D), the effect of monovalent ionic strength on the initial incision rates at the two monovalent salt concentrations can be calculated. Table II displays the ratio of endonuclease V initial incision rates at the different salt concentrations (rate at 100 mM KCl/rate at 10 mM KCl). The ratio of distributive versus processive initial incision rates provides a value by which comparisons which are independent of specific activity can be made. Table II also contains data from Nickell et al. (1991) regarding the A30K,V31L mutant

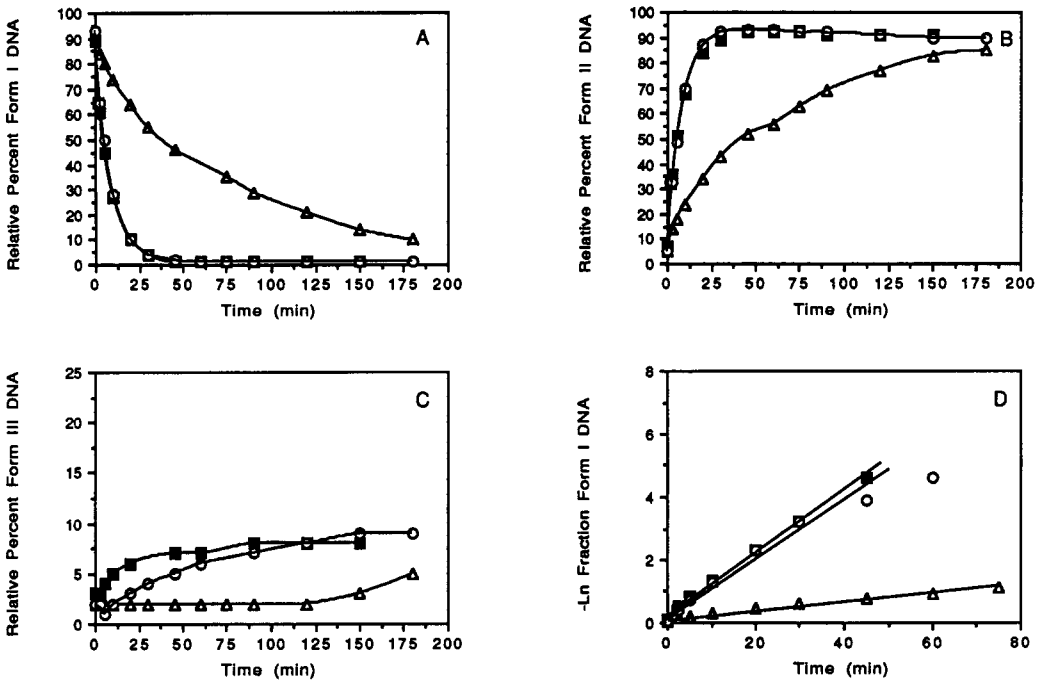


FIGURE 2: Kinetic analysis, under processive conditions, of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers. Partially purified endonuclease V was added to 1.0 μg of UV-irradiated [^{3}H]pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C and the reactions terminated at the indicated time. The three topological forms of DNA were separated on a 1% agarose gel, and their relative mass fraction was determined by scintillation spectroscopy. All experiments were performed in duplicate. Panel A shows the loss of form I DNA. Panel B shows the accumulation of form II DNA. Panel C shows the accumulation of form III DNA. Panel D shows the rate of disappearance of form I DNA. Endonuclease V (wild type), 1.0 ng/time point (■); A30K,V31L,N37K, 1 ng/time point (▲); A30K,V31L,H34K,N37K, 1 ng/time point (○).

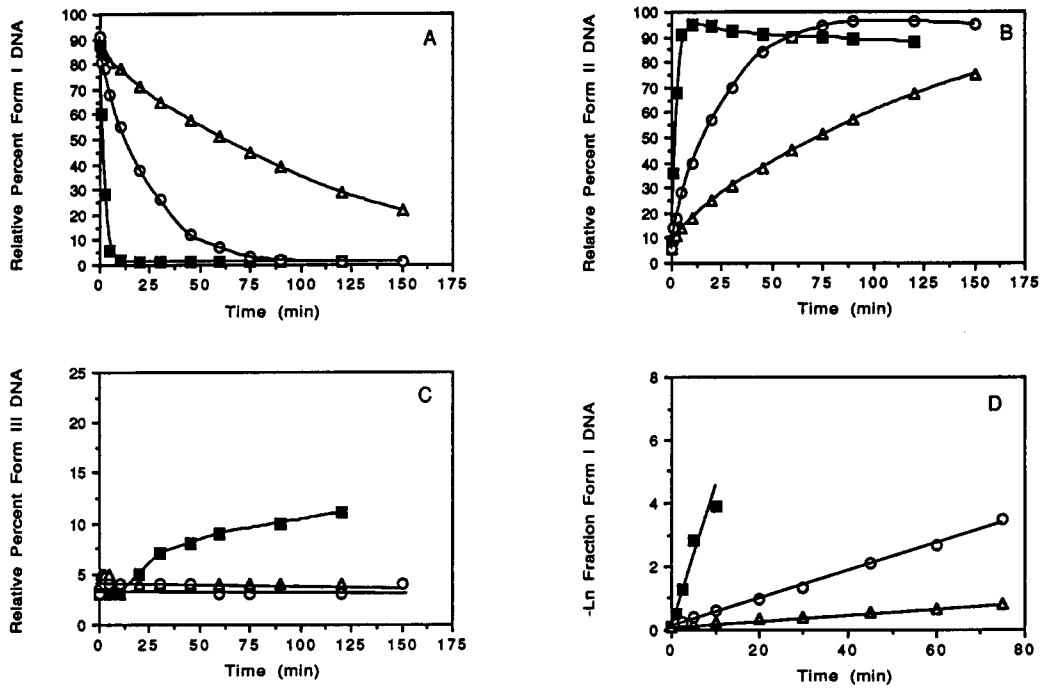


FIGURE 3: Kinetic analysis, under distributive conditions, of T4 endonuclease V nicking of form I DNA containing pyrimidine dimers. Partially purified endonuclease V was added to 1.0 μg of UV-irradiated [^{3}H]pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C for the indicated time. The three topological forms of DNA were separated on a 1% agarose gel, and the amount of each form was determined by scintillation spectroscopy. All experiments were performed in duplicate. Panel A shows the loss of form I DNA. Panel B shows the accumulation of form II DNA. Panel C shows the accumulation of form III DNA. Panel D shows the rate of disappearance of form I DNA. Endonuclease V (wild type), 0.1 ng/time point (■); A30K,V31L,N37K, 0.1 ng/time point (▲); A30K,V31L,H34K,N37K, 0.1 ng/time point (○).

enzyme. These results demonstrate that the differential magnitude of the initial incision rates of the mutant enzymes at the two ionic strength extremes decreased as the net positive charge increased. These results support our previous data which suggested that a series of mutant proteins have been created which display a gradient of enhanced nontarget DNA binding and, consequently, have initial incision reaction rates which are less affected than wild-type endonuclease V by increases in the salt concentration.

These kinetic analyses also monitor the accumulation of

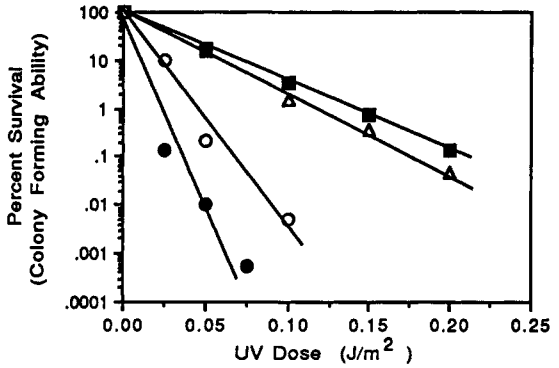


FIGURE 4: Colony-forming ability of UV-irradiated repair-deficient *E. coli* cells containing various *den*V gene constructs. AB2480 with pGX2608-*den*V$^-$ (●); pGX2608-*den*V$^+$ (■); pGX2608-*den*V-A30K,V31L,N37K (▲); pGX2608-*den*V-A30K,V31L,H34K,N37K (○).

form III DNA and, thus, indicate the mechanism of target search utilized by each enzyme. Under processive conditions at 10 mM KCl (Figure 2), the A30K,V31L,N37K mutant was observed to nick form I DNA at a reduced velocity compared to that of wild-type endonuclease V. The A30K,V31L,-H34K,N37K mutant had a pyrimidine dimer-specific nicking activity similar to that of wild-type endonuclease V. As expected under these processive conditions, form III DNA accumulated linearly in the presence of wild-type endonuclease V (panel C). Although the A30K,V31L,N37K mutant enzyme had a high nontarget DNA affinity, there appeared to be a lag in form III accumulation which is not consistent with a fully processive target search. However, a comparison of the accumulation of form III DNA between this mutant enzyme and wild-type endonuclease V when 10% form I DNA was remaining revealed a similar frequency of double-strand break formation. Although the A30K,V31L,H34K,N37K mutant had an initial incision rate similar to that of wild-type endonuclease V, the accumulation of form III DNA, as generated by this mutant, differed from that of wild-type endonuclease V. It appeared that there was a slight lag in the accumulation of form III DNA associated with this mutant, as though the mutant enzyme were utilizing a distributive search mechanism.

When the monovalent ionic strength conditions of the kinetic analysis are increased to 100 mM KCl, the process by which wild-type endonuclease V locates pyrimidine dimers within the UV-irradiated plasmid population in vitro is shifted to a distributive mechanism (Figure 3). The wild-type lag in form III DNA accumulation expected during a distributive reaction is clearly shown (panel C). The lag in form III accumulation for the two mutants was greatly exaggerated (to the point of no form III accumulation under these time constraints) due to a significant reduction in their rate of form I DNA loss compared to wild type under these high-salt conditions.

Survival of DNA Repair-Deficient Cells Expressing Mutant denV Genes. The survival of repair-deficient *E. coli* AB2480 (uvrA$^-$ recA$^-$) which had been transformed with plasmids expressing the mutant *den*V genes and subsequently irradiated with UV light for increasing time periods was measured (Figure 4). The expression of wild-type endonuclease V conferred a significant enhancement of UV resistance relative to cells containing only the parental vector pGX2608. Although the colony-forming ability of cells expressing the A30K,V31L mutant *den*V gene is not shown, the UV survival of this mutant was indistinguishable from that of wild-type endonuclease V (Nickell et al., 1991). The UV survival of cells expressing the A30K,V31L,N37K mutant *den*V gene was observed to be slightly less than the UV survival associated with wild-type *den*V expression. The UV survival associated with A30K,V31L,H34K,N37K mutant *den*V gene expression was even further reduced compared to wild-type survival. Despite this reduction, the colony-forming ability of cells expressing this latter mutant was clearly greater than that of cells harboring only the parental vector. There appears to be a gradient of UV survival associated with the net charge increase of the mutant enzymes. However, in this case, the gradient reflects a decrease in UV survival as the nontarget DNA binding increases. Since the two mutants described herein accumulated within *E. coli* at near-wild-type levels, mimicked wild-type purification character, and retained pyrimidine dimer-specific nicking activity, we believe it is unlikely that the reduced UV survival was the result of improper protein folding (Vershon et al., 1986) or disturbed protein homodimer formation.

Effect of Enhanced Nontarget DNA Binding Affinity on the Differential Substrate Specificities of T4 Endonuclease V Mutant Enzymes. There are several possible explanations for the observed reduced UV survival in light of our results which demonstrate a direct correlation between increased positive charge at our proposed DNA–protein interface and enhanced nontarget DNA binding affinity. It is possible that the enhanced attraction for nontarget DNA actually serves to increasingly retard or accelerate the one-dimensional diffusion of the A30K,V31L, the A30K,V31L,N37K and the A30K,V31L,H34K,N37K mutant enzymes under processive target search conditions. Alternatively, it is possible that the mutant enzymes' enhanced affinity for nontarget DNA actually alters their affinity for the pyrimidine dimer structure. As a result of this reduced affinity for the pyrimidine dimer structure, the mutants nonspecifically bind nontarget DNA and slide over and past pyrimidine dimer sites. To address these opposing interpretations, the AP-specific nicking activities of wild-type endonuclease V and the enhanced nontarget DNA binding mutants were compared to their respective pyrimidine dimer-specific nicking activities.

As stated earlier, the T4 endonuclease V repair mechanism is comprised of both DNA glycosylase and AP-lyase activities. These activities can be uncoupled both chemically and genetically. As a result, DNA containing AP sites, yet lacking pyrimidine dimer sites, may serve as a substrate for the AP-lyase activity of T4 endonuclease V. This activity results in the conversion of supercoiled form I DNA into nicked circular form II DNA. As shown in Figure 5 (panels A and B), the ability of the mutant enzymes to incise DNA containing AP sites was measured under processive conditions of 10 mM KCl as a function of increasing enzyme concentration. The AP incision rate associated with the two mutant enzymes was dramatically greater than that of wild-type endonuclease V. As the net positive charge increased, the rate of AP-specific cleavage increased 18-fold and 73-fold, respectively, by the A30K,V31L,N37K and the A30K,V31L,H34K,N37K mutants relative to the activity of wild-type endonuclease V.

The ability of wild-type endonuclease V and each of the mutant enzymes to incise UV-irradiated form I plasmid DNA was also evaluated under similar conditions as a function of increasing enzyme concentration (Figure 5, panels C and D). A reduced specific activity relative to that of wild type was observed with the A30K,V31L,N37K mutant enzyme. The form I DNA nicking activity of the A30K,V31L,H34K,N37K mutant enzyme was similar to that of wild-type endonuclease V. These results are in sharp contrast to the grossly altered AP-specific nicking activities of the two mutant enzymes.

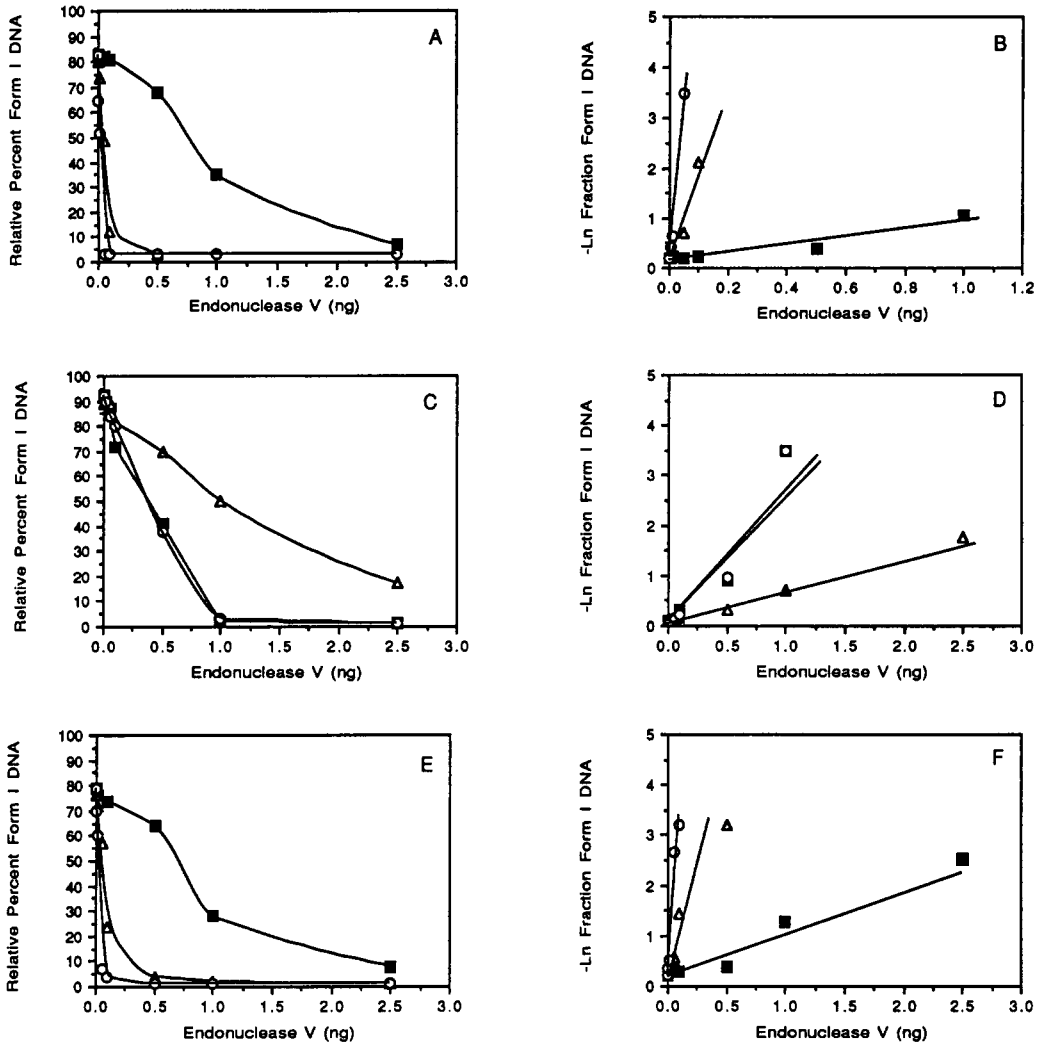


FIGURE 5: Differential substrate specificities of T4 endonuclease V mutant enzymes. (A) Partially purified endonuclease V was added to 1.0 μg of acid-depurinated [^{3}H]pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, and 1 mg/mL BSA. Solution were incubated at 37 °C for 30 min. The three topological forms of DNA were separated on a 1% agarose gel, and their relative mass fraction was determined by scintillation spectroscopy. (B) The data from (A) were transformed to a semilog plot, giving the first-order rate of loss of form I DNA. (C) Partially purified endonuclease V was added to 1.0 μg of UV-irradiated [^{3}H]pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C for 60 min. The three topological forms of DNA were separated on a 1% agarose gel, and their relative mass fraction was determined by scintillation spectroscopy. (D) The data from (C) were transformed to a semilog plot giving the first-order rate of loss of form I DNA. (E) Partially purified endonuclease V was added to 1.0 μg of acid-depurinated and UV-irradiated [^{3}H]pBR322 in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, and 1 mg/mL BSA. Solutions were incubated at 37 °C for 30 min. The three topological forms of DNA were separated on a 1% agarose gel, and their relative mass fraction was determined by scintillation spectroscopy. (F) The data from (E) were transformed to a semilog plot, giving the first-order rate of loss of form I DNA. Endonuclease V (wild type) (■); A30K,V31L,N37K (△); A30K,V31L,H34K,N37K (○). All experiments were performed in duplicate.

Finally, the ability of wild-type endonuclease V and each of the mutant enzymes to incise form I plasmid DNA which had been acid-depurinated and subsequently UV-irradiated was evaluated under processive conditions (10 mM KCl) as a function of increasing enzyme concentration (Figure 5, panels E and F). As the number of target pyrimidine dimer and AP sites is increased on a substrate DNA of fixed size, the nicking rate of wild-type endonuclease V would be expected to be less than the sum of the single substrate nicking rates due to its processive incision at each target site. True to this expectation, the rate of wild-type incision on a substrate containing both pyrimidine dimers and AP sites was reduced by 30% compared to the theoretical summation rate. However, the incision rates for the two mutants A30K,V31L,N37K and A30K,V31L,-H34K,N37K on the combination substrate were similar to the rates obtained on the substrate which had only been acid-depurinated (Figure 5, panels A and B). These mutants did not appear to be slowed by the addition of pyrimidine dimer sites. Rather, these data indicate that enhanced nontarget DNA affinity results in a reduced pyrimidine dimer-specific recognition and/or binding.

Discussion

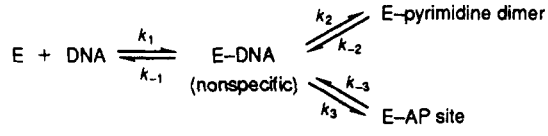
The theory of facilitated one-dimensional diffusion as discussed by Berg et al. (1981) predicts that a variation in the affinity of the protein for nontarget DNA will produce characteristic changes in the association and dissociation kinetics of the protein–target complex. In the case of T4 endonuclease V, it has been shown that a reduction in the electrostatic affinity of the enzyme for nontarget DNA, as modulated by increasing the salt concentration of the reaction environment and neutralizing key nontarget electrostatic interaction sites on the protein, leads to an increase in the nontarget DNA dissociation rate and a reduction in the processive character of the enzyme. Similarly, a destabilization of endonuclease V protein dimer formation also leads to in-

creased nontarget dissociation and decreased processivity.

In contrast, single positive charge increases at putative sites of endonuclease V–nontarget DNA interaction (at A30K, at H34K, and at N37K) have been shown to result in the formation of mutant enzymes which maintain high levels of processive nicking activity and display enhanced nontarget DNA affinities. Further incremental increases in the net positive charge along this protein–nontarget DNA interaction site, produced by the generation of the A30K,V31L,N37K and A30K,V31L,H34K,N37K mutants, have created altered enzymes which display a gradient of increasing affinity for nontarget DNA. This enhanced affinity was demonstrated by an expanded salt concentration over which unirradiated DNA competitively inhibits the incision of pyrimidine dimer-containing DNA. An additional manifestation of this enhanced nontarget DNA binding was shown to be a decreased rate of initial incision at pyrimidine dimer sites as a function of increasing monovalent ionic strength. Thus, these mutant's altered competition curves reflect the combination of enhanced nontarget DNA affinity and reduced pyrimidine dimer recognition and/or binding. Interestingly, these characteristics are inversely correlated with enhanced UV survival. As stated under Results, there are several possible explanations for this observation.

One possibility is that the increasing endonuclease V attraction for nontarget DNA inversely retards or accelerates the one-dimensional diffusion of the A30K,V31L, the A30K,V31L,N37K, and the A30K,V31L,H34K,N37K mutant enzymes under processive target search conditions. However, our results suggest that by increasing the endonuclease V attraction for nontarget DNA perhaps the ability of the A30K,V31L, the A30K,V31,N37K, and the A30K,V31L,-H34K,N37K mutant enzymes to recognize and/or bind the pyrimidine dimer structure is altered. As the pyrimidine dimer-specific binding affinity decreases, the rate of initial pyrimidine dimer incision falls. As pyrimidine dimers are missed during the mutant linear diffusion, a lag in form III DNA accumulation is observed, and UV survival is reduced.

If the endonuclease V reaction mechanism represented as



(where E = endonuclease V) is correct, then the observed increase in the AP-specific nicking activity of these mutants can be explored as a function of equilibrium constants. For wild-type endonuclease V, K_{dimer} and $K_{AP} >>> K_{nonspecific}$. However, we observe with the mutant enzymes presented in this paper that enhancing nontarget DNA affinity (reducing k_{-1}) reduces this difference. The increase in $K_{nonspecific}$ may be a contributing factor to the enhanced AP-specific nicking activity observed, yet the magnitude of this contribution will be diffusion-limited. Mechanistically, it is not likely that DNA scanning nor the association constants k_2 and k_3 have been altered by the enhanced nontarget DNA binding affinity. Thus, the observed reduction in the relative affinity of these mutant enzymes for a pyrimidine dimer versus the AP site is most likely the result of an alteration in the dissociation constants k_{-2} and k_{-3}. If k_{-2} is increased and k_{-3} is decreased, the increased dissociation from pyrimidine dimer sites relative to AP sites makes nontarget DNA a competitive inhibitor of pyrimidine dimer incision (Figure 1). The cumulative effect of the changes in these dissociation constants is manifested as an accelerated rate of AP site location and incision (Figure 5). Thus, these mutant's altered competition curves reflect the combination of enhanced nontarget DNA affinity and reduced pyrimidine dimer recognition and/or binding.

Wild-type endonuclease V is known to locate pyrimidine dimers via a DNA-scanning mechanism under low-salt conditions. Upon pyrimidine dimer binding, the enzyme cleaves the 5′-glycosylic bond of the pyrimidine dimer. The enzyme can then dissociate or go on to cleave the phosphodiester backbone between the two pyrimidines. It has not been shown that endonuclease V utilizes a DNA-scanning mechanism under low-salt conditions to locate AP sites generated by an unconcerted endonuclease V reaction or otherwise. Despite this lack of key data, the activity of these enhanced nontarget DNA binding mutants on AP-containing DNA is informative. If a retarded linear diffusion is the result of enhanced nontarget DNA affinity, then the activity of the endonuclease V mutants on either a pyrimidine dimer-containing or an AP-containing DNA substrate should be similar. At low-salt concentrations, this scenario predicts a reduced rate of pyrimidine dimer and AP incision which is independent of the site location mechanism. As shown in Figure 5, this is not the case. The AP-lyase activity of the endonuclease V mutants is greatly increased. Thus, the AP-lyase-specific incision data are consistent with the model asserting that enhanced nontarget DNA binding alters the mutant ability to recognize and/or bind pyrimidine dimers. These data also suggest, yet do not clearly demonstrate, that endonuclease V locates AP sites via a DNA-scanning mechanism under low salt conditions. When a substrate is produced containing both pyrimidine dimer sites and AP sites, only the wild-type enzyme experienced the expected decrease in the combined initial incision rate. The pyrimidine dimers appear to be overlooked by the mutants as they maintain their initial incision rate at the level observed when the substrate contained only AP sites.

The endonuclease V-enhanced electrostatic affinity studies were naively initiated with the goal of creating a more efficient repair enzyme. We accomplished just the opposite. Sines et al. (1990) suggest that alterations in protein point charge can have both general and specific effects. As the net charge of endonuclease V is increased along the putative endonuclease V–nontarget DNA interface, the general effect appears to be the desired enhanced nontarget DNA affinity. We consider it likely that a specific structure or point charge alteration effect is responsible for the undesired reduction in pyrimidine dimer recognition and/or binding activity. As changes in individual point charges enhance nontarget DNA affinity, each produces only very small changes in the structure or charge of the protein that is necessary to recognize and/or bind the kinked pyrimidine dimer structure (Pearlman et al., 1985; Husain et al., 1988). However, their cumulative effect ends with a protein structure or charge deformation which subtly alters the ability of the protein to recognize and/or bind its damaged substrate. The ultimate end is a less efficient repair enzyme.

The ability to efficiently locate and incise at the site of a pyrimidine dimer is of biological significance. DNA scanning, as defined by the one-dimensional facilitated diffusion of endonuclease V while nonspecifically bound to DNA, permits a greater sampling of DNA sequences per enzyme–DNA encounter and thus results in an increased rate of target location. The substitution of neutral amino acids for positively charged residues at endonuclease V positions Arg-3, Arg-26, and Lys-33 results in a reduced nontarget DNA affinity, a decreased nontarget DNA scanning capacity, and a subsequent reduced rate of target location. A decrease in the rate of target

location is correlated with a decrease in UV survival. At the other end of the spectrum, the enhanced nontarget DNA affinity of the endonuclease V mutants discussed in this paper results in an inefficient DNA scanning behavior and a subsequent reduced rate of effective target location. Again, the decrease in the rate of effective target location is correlated with a decrease in UV survival. Thus, it appears likely that wild-type endonuclease V reflects genetically-selected optimal nontarget and target DNA affinities that are necessary for maximal processive efficiency and survival. DNA scanning is undoubtedly a complex mechanism which involves delicate endonuclease V–nontarget DNA electrostatic interactions, high pyrimidine dimer-specific recognition and binding activity, and proper oligomeric enzyme structure.

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Acetyl Coenzyme A Binding by Chloramphenicol Acetyltransferase: Long-Range Electrostatic Determinants of Coenzyme A Recognition†

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ABSTRACT: The possible involvement of arginyl and lysyl side chains of chloramphenicol acetyltransferase (CAT) in binding coenzyme A (CoA) was studied by means of chemical modification, site-directed mutagenesis, variation in ionic strength, use of competitive inhibitors or substrate analogues, and X-ray crystallography. Unlike a number of enzymes, including citrate synthase, CAT does not employ specific ion pairs with the phosphoanionic centers of CoA to bind the acetyl donor, and arginyl residues play no role in recognition of the coenzyme. Although phenylglyoxal inactivates CAT reversibly, it does so by the formation of an unstable adduct with a thiol group, that of Cys-31 in the chloramphenicol binding site. The inhibitory effect of increasing ionic strength on k_{cat}/K_m(acetylCoA) can be explained by long-range electrostatic interactions between CoA and the ϵ-amino groups of Lys-54 and Lys-177, both of which are solvent-accessible. The ϵ-amino group of Lys-54 contributes 1.3 kcal·mol^{-1} to the binding of acetyl-CoA via interactions with both the 3′- and 5′-phosphoanions of CoA. Lys-177 contributes only 0.4 kcal·mol^{-1} to the productive binding of acetyl-CoA, mediated by long-range (~14 Å) interactions with the 5′-α- and -β-phosphoanions of CoA. The combined energetic contribution of Lys-54 and Lys-177 to acetyl-CoA binding (1.7 kcal·mol^{-1}) is less than that previously demonstrated (2.4 kcal·mol^{-1}) for a simple hydrophobic interaction between Tyr-178 and the adenine ring of CoA (Day & Shaw, 1992). In contrast to citrate synthase, the only other CoA binding enzyme for which high-resolution structural information is available, CAT recognizes CoA mainly by hydrophobic and polar (but uncharged) interactions.

Chloramphenicol acetyltransferase (CAT; EC 2.3.1.28)[1] is the enzyme responsible for high-level bacterial resistance to chloramphenicol (Shaw, 1983; Shaw & Leslie, 1991). The acetyl coenzyme A-dependent enzymic acetylation of chloramphenicol yields 3-acetylchloramphenicol which fails to bind to prokaryotic ribosomes (Shaw & Unowsky, 1968) and thus is devoid of antibiotic activity. Although CAT catalyzes the hydrolysis of acetyl-CoA in the absence of chloramphenicol, the thioesterase activity is 3 orders of magnitude lower than that of acetyl transfer to the antibiotic (Kleanthous & Shaw, 1984). Of more than a dozen naturally occurring CAT variants which have been described, only the type III enzyme has been studied in detail. The structure of CAT_{III}[2] is that of a homomeric trimer (3 × 25 kDa) with each of its three active sites lying deep in the clefts between subunits (Leslie et al., 1988; Leslie, 1990). The substrates approach the active site from opposite faces of the trimer to form a ternary complex, consistent with steady-state kinetic studies which revealed a sequential mechanism with a random order of addition of substrates (Kleanthous & Shaw, 1984).

Many enzymes which bind anionic substrates or cofactors have been shown to contain critical arginyl residues in their ligand binding sites (Riordan et al., 1977). Coenzyme A is such a cofactor, and on the basis of the results of chemical modification experiments with arginyl-specific reagents, it has been suggested that a number of enzymes bind CoA via ionic interactions with one or more arginyl residues (Mautner et al., 1981; Ramakrishna & Benjamin, 1981; Ragione et al.,

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM sodium chloride and 0.1 mM EDTA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CoA, coenzyme A; AcCoA, acetyl-CoA; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] Alignment of the amino acid sequences of seven CAT variants has resulted in a general numbering system which is used here. The numbering is related to the CAT_{III} linear sequence by subtracting 5 from residues 6-74 and 6 from residues 75-219 (Murray et al., 1988).

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