δ-Elimination by T4 Endonuclease V at a Thymine Dimer Site Requires a Secondary Binding Event and Amino Acid Glu-23†

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ABSTRACT: Endonuclease V from bacteriophage T4 is a well characterized enzyme that initiates the repair of ultraviolet light induced pyrimidine dimers. Scission of the phosphodiester backbone between the pyrimidines within a dimer, or 3′ to an abasic (AP) site, occurs by a β-elimination mechanism. In addition, high concentrations of endonuclease V have been reported to catalyze the cleavage of the $C_{5'}$−O−P bond in a reaction referred to as δ-elimination. To better understand the enzymology of endonuclease V, the δ-elimination reaction of the enzyme has been investigated using an oligonucleotide containing a site-specific *cis*−*syn* cyclobutane thymine dimer. The slower kinetics of the δ-elimination reaction compared to β-elimination and the ability of unlabeled dimer-containing DNA to compete more efficiently for δ-elimination than β-elimination indicate that δ-elimination most likely occurs during a separate enzyme encounter with the incised DNA. Previous studies have shown that both the α-amino group of the N-terminus and the acidic residue Glu-23 are necessary for the *N*-glycosylase and AP lyase activities of endonuclease V. Experiments with T2P, E23Q, and E23D mutants, which are defective in pyrimidine dimer-specific nicking, demonstrated that δ-elimination requires Glu-23, but not the primary amine at the N-terminus. In fact, the T2P mutant was much more efficient at promoting δ-elimination than the wild-type enzyme. Besides lending further proof that δ-elimination requires a second encounter between enzyme and DNA, this result may reflect an enhanced binding of the T2P mutant to dimer-containing DNA.

Endonuclease V of bacteriophage T4 is a small 16 kDa *N*-glycosylase/AP[1] lyase that initiates the repair of UV light-induced pyrimidine dimers. This enzyme has been extensively studied and is a good mechanistic model for the general class of glycosylase/AP lyase enzymes, which include the *Escherichia coli* enzymes Fpg and endonuclease III and the *Micrococcus luteus* UV endonuclease (Lloyd & Linn, 1993). Endonuclease V locates a pyrimidine dimer by a salt-sensitive one-dimensional diffusion along DNA (Lloyd et al., 1980; Gruskin & Lloyd, 1986, 1988; Ganesan et al., 1986; Dowd & Lloyd, 1989a,b). Upon binding its substrate, the enzyme catalyzes the removal of the 5′-pyrimidine of the dimer, followed by scission of the phosphodiester backbone 3′ to the newly created abasic (AP) site (Seawell et al., 1980; McMillan et al., 1981; Nakabeppu et al., 1982). The phosphodiester bond cleavage is the result of β-elimination, which leaves an α,β-unsaturated aldehyde on the 3′ terminus of the nicked DNA strand (Kim & Linn, 1988; Bailly et al., 1989a; Manoharan et al., 1988; Mazumder et al., 1989). Both *N*-glycosylase and AP lyase activities are initiated by the attack of the N-terminal amino group of endonuclease V on C-1′ of the 5′-deoxyribose moiety within the dimer (Schrock & Lloyd, 1991; Dodson et al., 1993). These two activities also require the presence of an additional residue, Glu-23 (Hori et al., 1992; Doi et al., 1992; Manuel et al., 1995).

Another enzyme belonging to the general class of glycosylase/AP lyase enzymes is the Fpg protein of *E. coli*. The Fpg protein, which has substrate specificity for 8-oxoguanine (8-oxoG) and formamidopyrimidine (Fapy) lesions (Tchou et al., 1991, 1994; Boiteux et al., 1992), has been shown to cleave the phosphodiester backbone both 3′ (β-elimination) and 5′ (δ-elimination) to an AP site, leaving a gap limited by 3′- and 5′-phosphate termini (Bailly et al., 1989b; O'Connor & Laval, 1989; Boiteux et al., 1990). Although most reports claim endonuclease V cleaves the phosphodiester bond only 3′ to an AP site (β-elimination), Bailly and co-workers (1989a) have reported that, at high concentrations, endonuclease V can also cleave the phosphodiester bond 5′ to an AP site. This secondary reaction, termed δ-elimination, is hypothesized to be similar to the β,δ-elimination reaction catalyzed by Fpg, although there is no evidence indicating whether the $C_{5'}$−O−P bond scission catalyzed by endonuclease V is due to an elimination or a hydrolytic mechanism.

In this study, we have characterized the δ-elimination reaction of endonuclease V on pyrimidine dimer-containing DNA in hopes of better understanding its enzymology. Furthermore, we have examined the ability of endonuclease V mutants deficient in *N*-glycosylase activity to catalyze the δ-elimination reaction to determine whether or not the same active sites that are important for the *N*-glycosylase/AP lyase reaction are also used for δ-elimination.

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[1] Abbreviations: UV, ultraviolet; AP, apurinic/apyrimidinic; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; CS 49-mer, *cis*−*syn* cyclobutane dimer adducted 49-base oligodeoxynucleotide.

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MATERIALS AND METHODS

Materials

Wild-type endonuclease V was purified from *E. coli* AB2480 (*uvrA*$^-$, *recA*$^-$) cells transformed with a *denV*-containing expression vector as previously described (Prince et al., 1991). The T2P, E23Q, and E23D mutants were created by site-directed mutagenesis in an M13mp18 construct containing the *denV* gene. The mutant genes were then subcloned into an expression vector which was used to transform *E. coli* AB2480. The T2P mutant was created and purified as reported (Schrock & Lloyd, 1993), and the E23Q and E23D mutants were made and isolated as described (Manuel et al., 1995). All proteins were found to be free from contaminants as demonstrated by silver staining of SDS−PAGE gels (data not shown). Oligonucleotide sizing markers were purchased from Pharmacia.

Methods

Preparation of Oligonucleotide Substrates. A 49-base oligodeoxynucleotide containing a site-specific *cis*−*syn* cyclobutane thymine dimer (Smith & Taylor, 1993) was the generous gift of C. A. Smith and J.-S. Taylor (Washington University, St. Louis, MO). This oligonucleotide had the sequence 5′-AGCTACCATGCCTGCACGAATTAAGCAAT-TCGTAATCATGGTCATAGCT-3′, where the underlined bases represent the position of the thymine dimer. The 49-mer was ^{32}P-labeled on the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP and then annealed to its complementary sequence to form the double-stranded substrate (CS 49-mer) used in subsequent reactions.

An abasic (AP) site containing substrate was created by reaction of a ^{32}P labeled 49-base duplex oligonucleotide (with the same sequence as the CS 49-mer, except that the thymine dimer was replaced with a uracil followed by a normal thymine residue) with *E. coli* uracil DNA glycosylase (Epicentre Technologies) as described previously (Latham et al., 1994).

Assays with Wild-Type Endonuclease V. Wild-type endonuclease V was reacted with the labeled CS 49-mer substrate (0.5 ng) for 30 min at 37 °C in the reaction buffer [25 mM sodium phosphate (pH 6.8), 1 mM EDTA, 25 mM KCl, and 100 μg/mL bovine serum albumin (BSA)]. This reaction product was then either left untreated or subjected to treatment with 1 M freshly diluted piperidine at 90 °C for 30 min. After removal of the piperidine by drying under vacuum, loading buffer [95% formamide, 20 mM EDTA, 0.025% (w/v) bromphenol blue, and 0.025% (w/v) xylene cyanol] was added, and the reaction products were separated by urea-containing polyacrylamide gel electrophoresis (PAGE). The AP 49-mer (0.37 ng) was also treated with piperidine in a similar manner, and the samples were loaded on the same gel.

For analysis of the concentration dependence of the reaction, the labeled CS 49-mer substrate (0.62 ng) was reacted with increasing amounts of endonuclease V in the reaction buffer. Reaction mixtures were incubated for 30 min at 37 °C and then terminated with the addition of electrophoresis loading buffer followed immediately by heating to 80 °C. The samples were separated by urea-containing PAGE, and the wet gels were subjected to autoradiography for visualization and phosphorimager analysis (Molecular Dynamics) for quantification.

To determine the time course of the reaction, the CS 49-mer substrate (0.50 ng) was allowed to react with 3.1 ng of endonuclease V in the reaction buffer at 37 °C for increasing amounts of time. Reactions were terminated and the samples were processed as described above.

Competition Experiments. To determine whether or not the presence of a competitor could influence the relative extent of the β- or δ-elimination reactions, unlabeled UV-irradiated plasmid DNA was used in the reactions between endonuclease V and the CS 49-mer. The 6.0 kb plasmid pGX2608-6 was UV-irradiated at 100 μW/cm^2 for 245 s to produce approximately 35 pyrimidine dimers per plasmid molecule (Lloyd et al., 1980). The CS 49-mer (0.62 ng), in the presence of increasing amounts of UV-irradiated plasmid DNA, was incubated at 37 °C for 30 min with 10.3 ng of endonuclease V in the reaction buffer. In a second experiment, reactions containing 0.50 ng of CS 49-mer and 3.1 ng endonuclease V were allowed to proceed for 1 min at 37 °C. After this initial incubation time, UV-irradiated plasmid DNA (6 μg), or water as a control, was added, and the reactions were allowed to proceed for 1, 4, or 9 additional min. Samples were analyzed as already described.

Assay To Determine the Competence of T2P, E23Q, or E23D Mutants in Catalyzing δ-Elimination. Radiolabeled CS 49-mer (0.77 ng) was incubated with 100 ng of wild-type, T2P, E23Q, or E23D endonuclease V in a reaction buffer [25 mM sodium phosphate (pH 6.8), 1 mM EDTA, 100 mM KCl, and 100 μg/mL bovine serum albumin (BSA)] for 15 min at 37 °C. The CS 49-mer was also reacted with 1 ng of wild-type endonuclease V, and these reactions were either terminated after 15 min or allowed to continue for an additional 15 min following the addition of buffer or 100 ng of wild-type, T2P, E23Q, or E23D endonuclease V. The samples were subjected to electrophoresis, autoradiography, and analysis by phosphorimager as already described.

Gel Mobility Shift Assay. To qualitatively analyze the ability of the T2P, E23Q, and E23D mutants to bind thymine dimer-containing DNA, 100 ng of each mutant was incubated for 15 min at 20 °C with 0.78 ng of the ^{32}P-labeled CS 49-mer in binding buffer [0.5× TBE, 5% (v/v) glycerol, 150 mM NaCl, 0.33 μg/mL poly(dI·dC), and 0.3% (w/v) bromphenol blue]. The complexes were then separated by electrophoresis through a nondenaturing 10% polyacrylamide gel and visualized by autoradiography.

RESULTS AND DISCUSSION

Endonuclease V from the bacteriophage T4, along with Fpg and endonuclease III from *E. coli* and the UV endonuclease from *M. luteus* are *N*-glycosylases with associated AP lyase activities. Endonuclease V, endonuclease III, and the *M. luteus* UV endonuclease leave 3′ α,β-unsaturated aldehyde termini after phosphodiester bond cleavage. These enzymes have been shown to cleave DNA at AP sites via a β-elimination mechanism (Lloyd & Linn, 1993; Kim & Linn, 1988; Bailly et al., 1989a; Manoharan et al., 1988; Mazumder et al., 1989). In contrast, the Fpg protein catalyzes a β,δ-elimination reaction at AP sites, leaving a 3′-phosphate end (Bailly et al., 1989b; O'Connor & Laval, 1989; Boiteux et al., 1990). In addition to the normal β-elimination product, high concentrations of endonuclease V have been shown to generate a small amount of cleaved product terminating in a 3′-phosphate after reaction

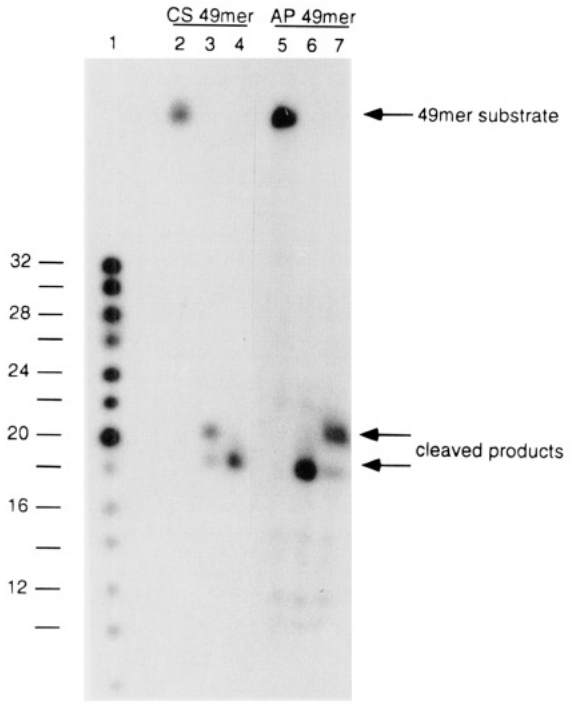

FIGURE 1: Endonuclease V cleaves the CS 49-mer to produce both a β-elimination product and a δ-elimination product. The ^{32}P-labeled CS 49-mer and AP 49-mer were prepared as described in Material and Methods. The endonuclease V reactions were carried out for 30 min at 37 °C, and the piperidine treatment was performed by adding freshly diluted piperidine to a final concentration of 1 M and heating to 90 °C for 30 min. Lane 1, oligonucleotide sizing markers (8−32 bases) where the numbers correspond to the number of bases; lane 2, CS 49-mer alone; lane 3, CS 49-mer reacted with endonuclease V; lane 4: CS 49-mer reacted with endonuclease V and then treated with piperidine; lane 5, AP 49-mer; lane 6, AP 49-mer plus piperidine; lane 7, AP 49-mer reacted with endonuclease V.

with AP site or thymine dimer containing DNA (Bailly et al., 1989a). This "δ-elimination" product results from cleavage of the $C_{5'}$−O−P bond 5′ to the AP site. We have examined the δ-elimination reaction of endonuclease V to lend insight into the overall reaction mechanism of glycosylase/AP lyases and into the specific interaction of endonuclease V with dimer-containing DNA.

The δ-Elimination Product Has a 3′-Phosphate Terminus. Endonuclease V was allowed to react with a double-stranded oligonucleotide containing a site-specific *cis*−*syn* cyclobutane thymine dimer (CS 49-mer) which had been ^{32}P-labeled at the 5′ terminus of the dimer-containing strand. The reaction products were analyzed by urea-containing polyacrylamide gel electrophoresis (PAGE). Two product bands were evident after endonuclease V cleavage of this substrate, the slower and more prominent of which migrated just above the 20-mer marker (Figure 1, lane 3). The major band had a mobility consistent with the expected β-elimination product, which is known to terminate in a 3′ α,β-unsaturated aldehyde (Kim & Linn, 1988; Bailly et al., 1989a; Manoharan et al., 1988; Mazumder et al., 1989). The second product band, with a mobility just faster than the 20-mer marker, was consistent with the δ-elimination product first identified by Bailly et al. (1989a), which has a 3′-phosphate terminus. To prove that this faster mobility band was due to a 20-mer terminating in a 3′-phosphate, and thus was the same product identified by Bailly et al. (1989a), piperidine was added after the endonuclease V reaction. After piperidine treatment, a single band was evident with a mobility identical to the faster moving endonuclease V product (Figure 1, lane 4). Piperidine reacts with the C-1′ of an AP site or a β-elimination product to cause scission of the $C_{5'}$−O−P bond, leaving a cleaved oligonucleotide with a 3′-phosphate terminus. Because the mobility of the piperidine cleaved product was identical to the faster mobility product of the endonuclease V, this faster endonuclease V-derived DNA product was concluded to terminate in a 3′-phosphate. As further proof as to the nature of this band, an authentic AP site containing oligonucleotide (AP 49-mer) was prepared and reacted with piperidine. Again, the single piperidine cleavage product migrated at the same rate as the faster mobility band formed by the endonuclease V reaction with the CS 49-mer (Figure 1). In addition, reaction of the AP 49-mer with endonuclease V produced two bands, and the faster mobility band migrated at the same rate as the product of the piperidine reaction with the AP 49-mer. These data indicate that this faster migrating band does indeed have a 3′-phosphate terminus and thus is identical to the δ-elimination product identified by Bailly and co-workers (1989a).

Concentration Dependence of δ-Elimination. We investigated the relationship between endonuclease V concentration and the amount of δ-elimination product formed using the same 49-base pair oligonucleotide with a single thymine dimer site. Increasing amounts of endonuclease V were reacted with the CS 49-mer which had been ^{32}P-labeled at the 5′ terminus of the dimer-containing strand. The reaction products were analyzed by urea-containing PAGE (Figure 2A). The enzyme was in a 10-fold molar excess over substrate before substantial amounts of the δ-elimination product were visible by autoradiography (Figure 2A, lane 7). Furthermore, the observed amount of this product, as quantitated by phosphorimager analysis, was small until virtually all of the CS 49-mer reactant was converted to the β-elimination product (Figure 2B). At the highest concentration used, which represented a 1000-fold molar excess of enzyme to DNA, 26% of the total product was due to δ-elimination (Figure 2A, lane 11). At a ratio of 1:1 enzyme to DNA, only 8.4% of the product was from δ-elimination (Figure 2A, lane 5). The high ratios of enzyme to DNA necessary to produce significant amounts of the δ-elimination product (e.g., a 10:1 ratio of enzyme to substrate caused 20% of the product to migrate with the δ-elimination band) could indicate that the enzyme binds independently to the β-elimination product after an initial dissociation. The sharp increase in the amount of δ-elimination product formed between the 3.2 and 10 nM endonuclease V concentrations directly corresponds to a sharp increase in the amount of substrate cleaved (Figure 2B). This result could indicate that the β-elimination product is the substrate for the δ-elimination reaction. Alternately, the enzyme could catalyze δ-elimination in a fraction of all encounters with a thymine dimer site.

Time Course of δ-Elimination. To lend insight into the kinetics of the δ-elimination reaction, a time course assay was performed. If the δ-elimination reaction requires the enzyme to rebind the nicked pyrimidine dimer site after releasing the β-elimination product, it would be expected that the β-elimination product would accumulate much faster than the δ-elimination product, especially at the early time points. On the other hand, if endonuclease V remains at the cleaved dimer site before promoting δ-elimination, but

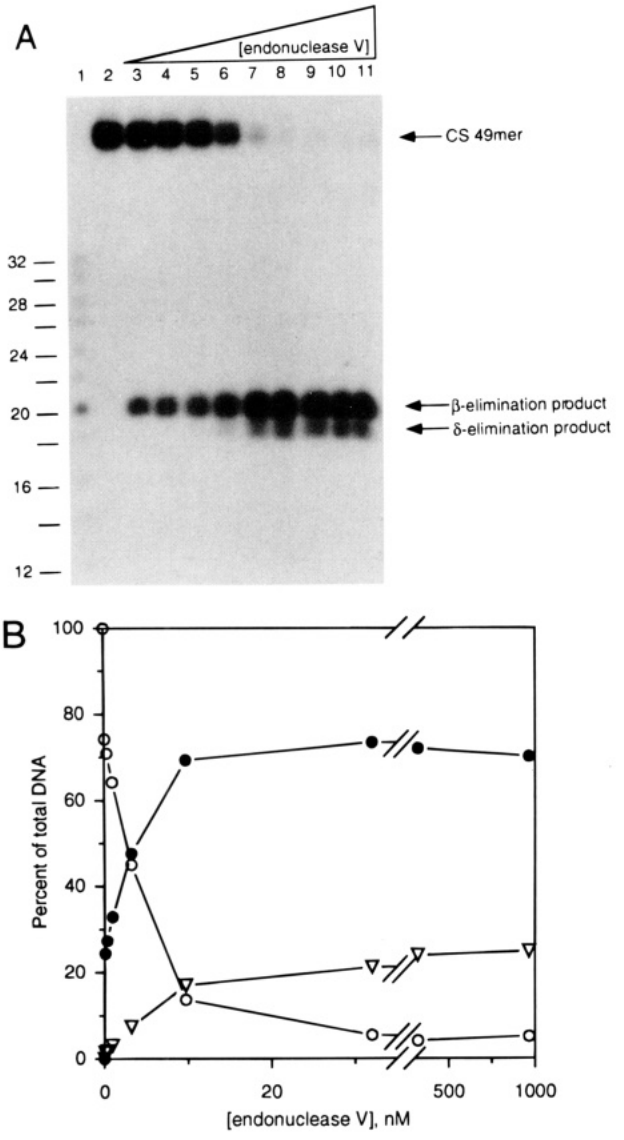

FIGURE 2: Dependence of δ-elimination on endonuclease V concentration. The CS 49-mer was ^{32}P-labeled on the 5′ terminus of the thymine dimer-containing strand and reacted with increasing concentrations of endonuclease V. The molar ratios of enzyme to DNA ranged from 1:10 at the lowest enzyme concentration to 1000:1 at the highest concentration. Reactions were terminated after a 30 min incubation at 37 °C, and the DNA was analyzed by polyacrylamide gel electrophoresis. (A) Autoradiogram of the polyacrylamide gel. Lane 1, oligonucleotide sizing markers; lane 2, CS 49-mer alone; lane 3, 0.097 nM endonuclease V; lane 4, 0.32 nM; lane 5, 0.97 nM; lane 6, 3.2 nM; lane 7, 9.7 nM; lane 8, 32 nM; lane 9, 97 nM; lane 10, 320 nM; lane 11, 970 nM endonuclease V. (B) The data were quantified with a phosphorimager and converted to a percentage of the total DNA. (○) Intact CS 49-mer; (●) β-elimination product; (▽) δ-elimination product.

only a fraction of those interactions actually allow δ-elimination to occur, there should be a constant ratio of β- to δ-elimination products evident over time.

The CS 49-mer was allowed to react with endonuclease V in a low-salt buffer for increasing amounts of time (Figure 3A,B). We have found that processive conditions, produced by low salt concentrations *in vitro*, greatly enhance the amount of δ-elimination product formed (data not shown). Phosphorimager analysis of the products indicated that there was not a significant lag in the formation of the δ-elimination band, although the β-elimination product was noted to accumulate at a much faster rate than the δ-elimination

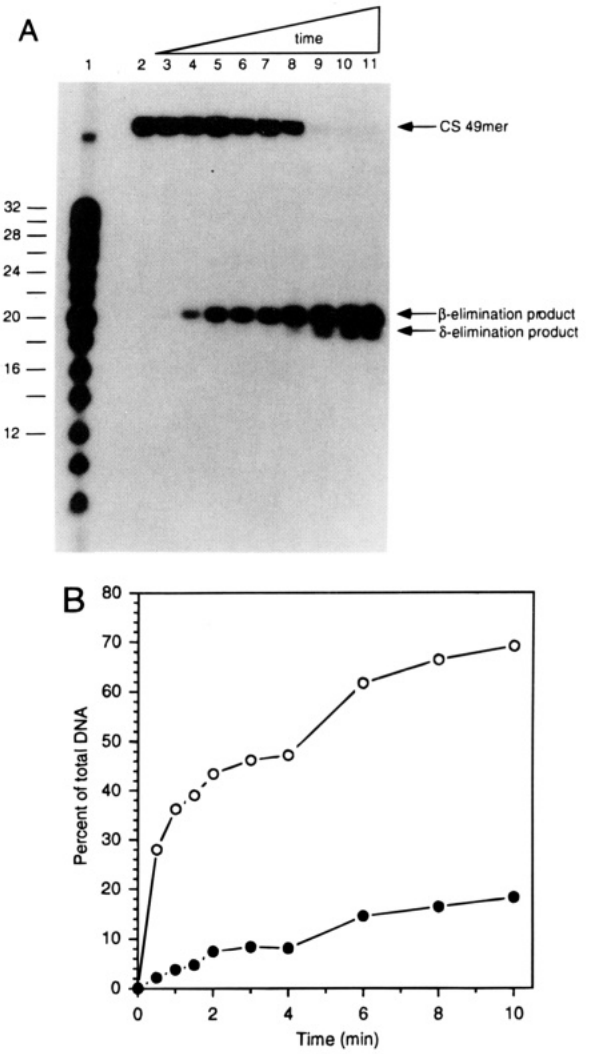

FIGURE 3: Time course of β- and δ-elimination reactions of endonuclease V. CS 49-mer (0.50 ng) was incubated with 3.1 ng (9.7 nM final concentration) of endonuclease V for increasing periods of time. (A) Autoradiogram of the polyacrylamide gel. Lane 1, oligonucleotide markers; lane 2, CS 49-mer alone; lane 3, 30 s; lane 4, 1 min; lane 5, 1.5 min; lane 6, 2 min; lane 7, 3 min; lane 8, 4 min; lane 9, 6 min; lane 10, 8 min; lane 11, 10 min. (B) Graph of the data as percent of total DNA. (○) β-Elimination product; (●) δ-elimination product.

product. At the 1 min time point, only 9.5% of the total product was due to δ-elimination, and the ratio of β- to δ-elimination product was 9.5:1. This distribution of products was in contrast to that at the later time points. For instance, after a 10 min reaction time, 79% of the product was due to β-elimination and 22% was due to δ-elimination, making the ratio of the β-elimination product to δ-elimination product 3.8:1. These data suggest that endonuclease V does not remain at the dimer site but rather reassociates with the β-elimination product to effect δ-elimination.

δ-Elimination Occurs after a Second Enzyme Encounter with DNA. To further investigate the idea that endonuclease V catalyzes δ-elimination by a separate binding event after first cleaving the phosphodiester bond by β-elimination, reactions were carried out in the presence of competitor DNA. Endonuclease V has been found by filter binding assay to bind to a thymine dimer site with a K_d of 2.0×10^{-8} M (Iwai et al., 1994), and since neither the enzyme−nontarget DNA complex nor the enzyme-cleaved DNA complex can be retained on a filter in this assay, the K_d has

been estimated to be at least an order of magnitude higher for both of these substrates than for an intact pyrimidine dimer site (Seawell et al., 1980; Inaoka et al., 1989). If the enzyme requires a secondary encounter with DNA to initiate δ-elimination, the addition of pyrimidine dimer-containing competitor DNA would be expected to affect δ-elimination to an even greater extent than β-elimination. This would result from the enzyme having a much higher affinity for an intact pyrimidine dimer than for a nicked dimer site. Endonuclease V was reacted with the CS 49-mer in the presence of increasing amounts of UV-irradiated plasmid DNA (Figure 4A). In the absence of plasmid DNA, 95% of the reactant was converted to product, and, of the products, 24% was due to δ-elimination. This ratio of δ- to β-elimination product greatly diminished with the addition of UV-irradiated plasmid DNA. For instance, when 1.2 μg of competitor was added (~400-fold molar excess of pyrimidine dimers over those contributed by the CS 49-mer), 80% of the CS 49-mer was cleaved but only 6.6% of the total product formed was due to δ-elimination (Figure 4A). These competition data can be explained if the enzyme catalyzes δ-elimination only after a separate interaction with the β-elimination product. In support of this idea, low salt conditions, which allow for a processive target search mechanism in vitro by increasing the enzyme's affinity for nontarget DNA, enhance the amount of δ-elimination product observed (data not shown).

In contrast to the second encounter hypothesis, a β,δ-elimination mechanism would require endonuclease V to remain at the cleaved pyrimidine dimer site long enough to catalyze δ-elimination. In this scenario, addition of competitor DNA after a short initial reaction period would be expected to bind free enzyme but allow any enzyme already bound to a thymine dimer to complete its reaction. If that reaction included δ-elimination, one should see an increase in the δ-elimination product even after the addition of competitor DNA. On the other hand, if the enzyme does require a secondary binding event to catalyze δ-elimination, then the addition of large amounts of competitor DNA would effectively halt further δ-elimination reaction. To test this possibility, the CS 49-mer was allowed to react with endonuclease V for 1 min. After this initial reaction time, either water or unlabeled UV-irradiated DNA was added to the reaction mixtures, and the samples were incubated for an additional time period (Figure 4B). The addition of DNA to the reactions effectively competed for both β- and δ-elimination reactions, although the competition was more pronounced for the δ-elimination reaction. After the addition of competitor followed by a 1 min incubation (Figure 4B, column D), 3.6% of the DNA was found in the δ-elimination band (9.5:1, β- to δ-elimination product) compared to 6.2% in the control reaction (7.9:1, β- to δ-elimination product; column C). This trend continued throughout all time points analyzed. In the 9 min reaction with competitor, only 5.7% of the DNA was in the δ-elimination product band, whereas, in the 9 min reaction in the absence of competitor, 16% of the total DNA was due to δ-elimination. Although these results are consistent with the secondary encounter hypothesis, where the enzyme catalyzes δ-elimination after releasing and then making a second encounter with the nicked DNA site, they could also reflect the fact that so few molecules of endonuclease V are bound to DNA when the competitor is added that any reaction of prebound enzyme would be

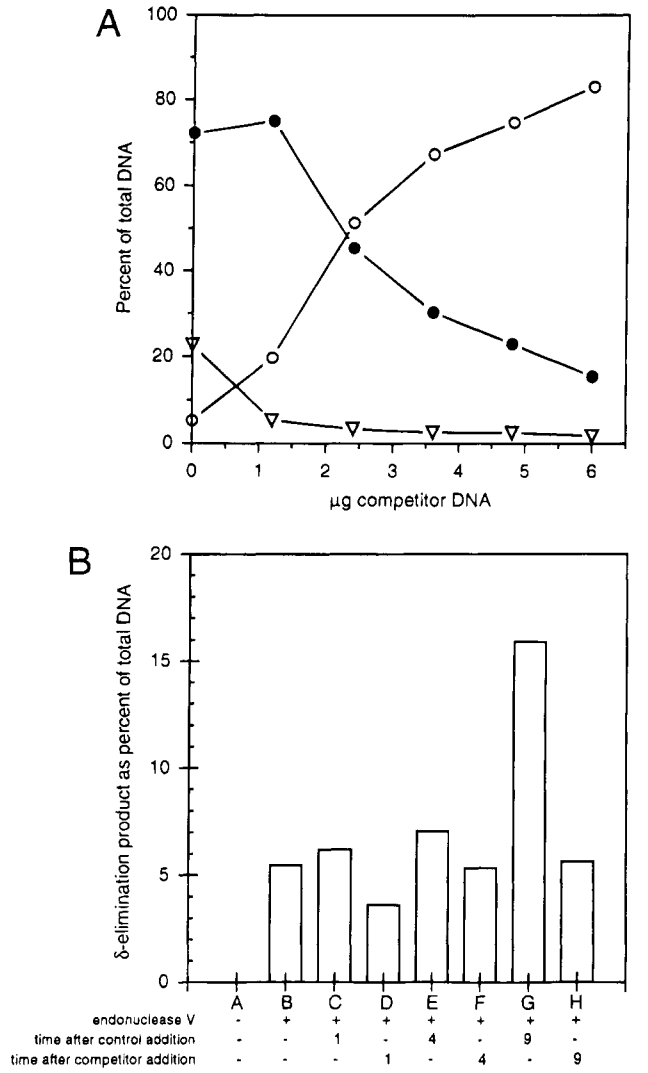

FIGURE 4: Competition of endonuclease V catalyzed cleavage of the CS 49-mer by UV-irradiated plasmid DNA. (A) ^{32}P-labeled CS 49-mer (0.62 ng) was reacted for 30 min at 37 °C with 10.3 ng of endonuclease V in the presence of increasing amounts of unlabeled UV-irradiated plasmid DNA. Data are shown as percent of total DNA. (○) intact CS 49-mer; (●) β-elimination product; (▽) δ-elimination product. (B) Unlabeled UV-irradiated plasmid DNA (6.0 μg) was added to samples containing 3.1 ng of endonuclease V and 0.50 ng CS 49-mer after an initial reaction period of 1 min. Water was added as the control. The reactions were allowed to proceed for 1, 4, or 9 additional min. The δ-elimination product as a percent of total DNA is depicted. (A) No reaction; (B) 1 min reaction only, no competitor added; (C and D) 1 min reaction followed by the addition of water or plasmid DNA, respectively, and an additional 1 min incubation; (E and F) 1 min reaction followed by the addition of water or plasmid DNA, respectively, and an additional 4 min incubation; (G and H) 1 min reaction followed by the addition of water or plasmid DNA, respectively, and an additional 9 min incubation.

impossible to detect. Since all experiments in this study point to the need for a secondary encounter between endonuclease V and the initially cleaved site, we feel we can discount this latter possibility.

Because a secondary interaction between endonuclease V and DNA nicked at a pyrimidine dimer site was found to be necessary for δ-elimination, it is conceivable that the δ-elimination reaction is not a specific enzymatic activity of endonuclease V. Endonuclease V is a highly basic protein (Valerie et al., 1984), and highly basic molecules, including polyamines and histones, are known to catalyze cleavage of

DNA at AP sites in a nonspecific manner (Bailly & Verly, 1988). To ensure that the δ-elimination reaction is catalyzed by endonuclease V in a specific manner, we reacted the β-elimination product with large molar excesses of both spermidine and the basic protein gp32 (a generous gift from the laboratory of Bruce Alberts, UCSF), which is a single strand binding protein of bacteriophage T4. Neither spermidine nor gp32 could catalyze the conversion of the β-elimination product to the δ-elimination product, even when added at concentrations 16 times higher than the highest wild-type endonuclease V concentration tested (data not shown). These data indicate that the δ-elimination reaction is a specific enzymatic activity of endonuclease V.

δ-Elimination by Mutant Enzymes Defective in Thymine Dimer-Specific Nicking. Experiments from this laboratory have demonstrated that the N-terminus of endonuclease V is directly responsible for both the *N*-glycosylase and AP lyase activities of the enzyme (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993). Furthermore, Glu-23, which has been shown by X-ray crystallography to be close to the N-terminus, has also been found to be catalytically important for both activities (Morikawa et al., 1992; Hori et al., 1992; Doi et al., 1992; Manuel et al., 1995). To test whether or not the αNH_2 group on the N-terminus or Glu-23 was necessary to promote δ-elimination, mutants at these two positions were tested for their ability to catalyze δ-elimination after initial reaction of the CS 49-mer with a small amount of wild-type endonuclease V. The T2P and E23Q mutants were previously shown to be unable to catalyze the glycosylase reaction at a pyrimidine dimer, while the E23D mutant retains only 1% of the normal glycosylase activity (Schrock & Lloyd 1993; Doi et al., 1992; Manuel et al., 1995). Furthermore, the E23Q mutant has been shown to have lost AP lyase activity, whereas the T2P and the E23D mutants retained partial AP lyase activity (Manuel et al., 1995; R. Schrock and R. S. Lloyd, unpublished observations). All three of these mutants were tested for their ability to catalyze δ-elimination.

A small amount of wild-type endonuclease V was used to convert the CS 49-mer to the β-elimination product. An excess of wild-type or mutant protein was then added to the reactions, and the products were examined by gel electrophoresis (Figure 5A). The E23Q and E23D mutants were not able to catalyze δ-elimination of the cleaved α,β-unsaturated aldehyde product (Figure 5A,B). The T2P mutant, on the other hand, catalyzed a very efficient removal of the 3′ sugar fragment. Indeed, when the T2P mutant was added after the limited initial reaction with wild-type endonuclease V, 64% of the product was found to be due to δ-elimination. In contrast to the T2P mutant, when an excess of the wild-type enzyme was added after the initial reaction period, only 18% of the product was due to δ-elimination. Interestingly, the T2P mutant was also able to convert AP sites to the δ-elimination product much more efficiently than the wild-type enzyme. This ability was not evident in the E23D mutant (data not shown). The fact that the addition of mutant endonuclease V to an already nicked substrate causes conversion of the preformed β-elimination product to the δ-elimination product provides further evidence that a secondary encounter with DNA occurs to promote the δ-elimination reaction. These results also demonstrate that the Glu-23 residue is important in the δ-elimination reaction,

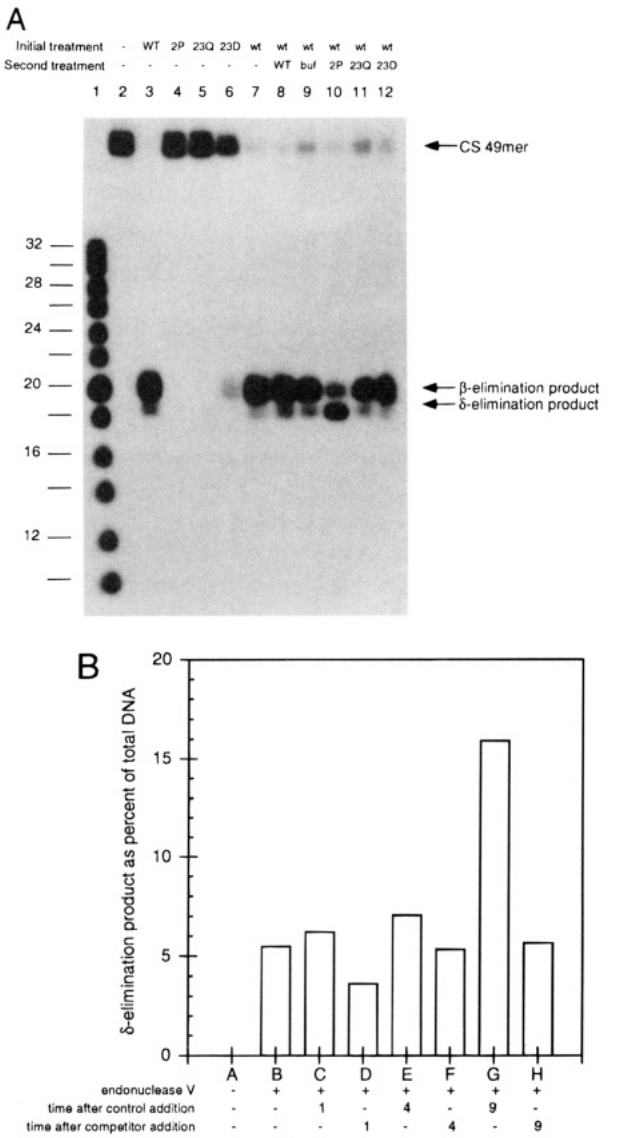

FIGURE 5: Ability of wild-type, T2P, E23Q, and E23D endonuclease V to catalyze δ-elimination from a β-elimination product. (A) The CS 49-mer (0.77 ng; lane 2) was reacted with 100 ng of wild-type (lane 3), T2P (lane 4), E23Q (lane 5), or E23D (lane 6) endonuclease V for 15 min at 37 °C. The CS 49-mer was also reacted with 1 ng of wild-type endonuclease V for 15 min (lane 7) followed by the addition of 100 ng of wild-type (lane 8), buffer (lane 9), 100 ng of T2P (lane 10), 100 ng of E23Q (lane 11), or 100 ng of E23D (lane 12) and an additional 15 min incubation. (B) Data from panel A were acquired by phosphorimager and expressed as the amount of δ-elimination product as a percent of the total cleavage product. (A) 100 ng of wild-type only; (B) 1 ng of wild-type only; (C−G) 1 ng of wild-type incubated for 15 min followed by C, 100 ng of wild-type; (D) buffer; (E) 100 ng of T2P; (F) 100 ng of E23Q; and (G) 100 ng of E23D.

whereas the presence of a primary amine situated at the N-terminus is not.

If endonuclease V makes a secondary encounter to catalyze δ-elimination, the affinity of the enzyme for a pyrimidine dimer or nontarget DNA would be expected to affect the rate of δ-elimination. A gel mobility shift assay was performed on the E23Q, E23D, and T2P mutants to examine their ability to bind the CS 49-mer. The wild-type enzyme cleaves the CS 49-mer substrate, so binding of wild-type endonuclease V cannot be studied using this technique. The gel shift experiments showed that the E23Q and E23D mutants bound to the CS 49-mer DNA to form two major

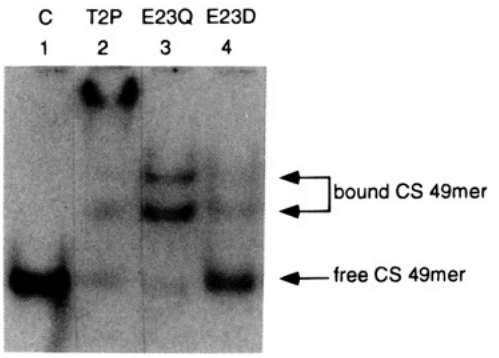

FIGURE 6: Ability of the T2P, E23Q, and E23D mutants of endonuclease V to bind the CS 49-mer. The CS 49-mer (0.78 ng) was incubated with 100 ng of each of the three mutants for 15 min at 20 °C, and the protein−DNA complexes were analyzed by electrophoresis through a nondenaturing polyacrylamide gel. Lane 1, CS 49-mer alone; lane 2, T2P; lane 3, E23Q; lane 4, E23D.

bands of retarded mobility (Figure 6, lanes 3 and 4). These two complexes have been shown to be consistent with one and two molecules of enzyme bound per oligonucleotide (Manuel et al., 1995). Studies with wild-type endonuclease V and both dimer-containing and tetrahydrofuran-containing oligonucleotides have indicated that a single molecule of wild-type binds per damaged site (manuscript in preparation). The T2P mutant, on the other hand, forms a large molecular weight complex with a much slower mobility than the E23Q and E23D mutant complexes. The presence of a high molecular weight complex with the T2P endonuclease V may reflect coating of the CS 49-mer by the mutant enzyme, perhaps due to a higher affinity of the T2P for DNA. If the T2P mutant actually binds DNA more tightly than wild-type, the T2P mutant may engage in a greater number of favorable interactions with the β-elimination product, allowing δ-elimination to occur more efficiently.

Mechanistic Insights. The "δ-elimination reaction" that results in the removal of the 3′ sugar fragment left from endonuclease V-catalyzed β-elimination at a pyrimidine dimer or AP site could actually be due to either an elimination or hydrolytic mechanism. The data presented herein indicate that endonuclease V releases the β-elimination products after thymine dimer specific nicking. The primary amino group at the N-terminus is not necessary for δ-elimination, as T2P catalyzes even more efficient δ-elimination than wild-type. The Glu-23 residue may be involved in the δ-elimination mechanism, perhaps by removing the 4′-H from the molecule, which would result in the elimination of the 5′-phosphate. A proposed mechanism for the action of endonuclease V is depicted in Figure 7. The protonation step is hypothesized, and further experimentation would be necessary to prove that this step occurs. If the "δ-elimination" is actually a consequence of hydrolysis, the Glu-23 residue could be activating a water molecule to initiate the removal of the 3′ sugar fragment. Positive identification of the sugar fragment released by the β- and δ-elimination

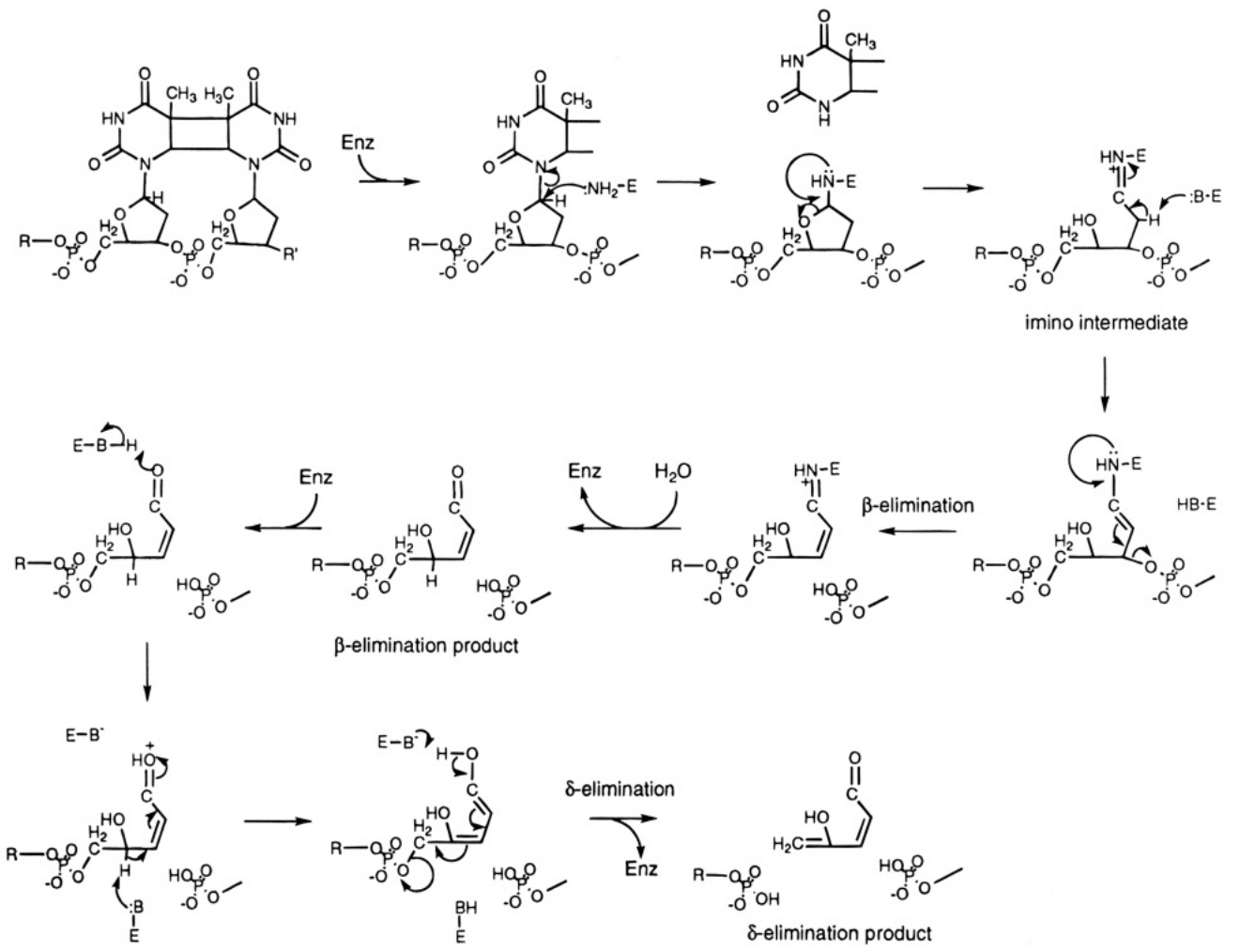

FIGURE 7: Proposed catalytic mechanism of the endonuclease V-mediated β-elimination and δ-elimination reactions at a thymine dimer site.

reactions or evidence that the 4′-H is removed from the β-elimination product is needed before the mechanism of this third catalytic activity of endonuclease V can be completely resolved.

Due to the high concentrations necessary for the δ-elimination reaction to occur, it is unlikely that this third catalytic activity of endonuclease V is important within a cell, although the *in vivo* situation is difficult to discern. The repair of damaged sites requires the presence of a 3′-hydroxyl moiety for polymerization and ultimate ligation of gaps. Neither the 3′-α,β-unsaturated aldehyde left by β-elimination nor the 3′-phosphate resulting from a β,δ-elimination event are substrates for DNA polymerases. These products, like the AP sites left by glycosylases without AP lyase activity, require further processing by AP endonucleases, such as endonuclease IV or exonuclease III of *E. coli*. It seems that phosphodiester bond scission catalyzed by glycosylase/AP lyase enzymes is a consequence of the *N*-glycosylase mechanism employed (Dodson et al., 1994). Furthermore, it is likely that evolution has preserved the AP lyase activity and perhaps the δ-elimination activity only because they correlate with efficient glycosylase action. For this reason, neither the AP lyase action nor the δ-elimination activity of endonuclease V may be biologically important, although examination of these events gives insight into the overall mechanism of the enzyme.

Study of the δ-elimination reaction has led to insight into the interaction of endonuclease V with pyrimidine dimer containing DNA. We now know that endonuclease V can dissociate from the nicked pyrimidine dimer and then reassociate to form a specific complex that allows for removal of the 3′ sugar fragment. We also know that one residue important for the *N*-glycosylase and AP lyase functions of the enzyme, Glu-23, also is influential in catalyzing δ-elimination. The study of the endonuclease V δ-elimination reaction may lend insight into the reaction mechanism of the Fpg protein, which normally catalyzes a β,δ-elimination reaction at its target sites. It is probable that one of the keys to the Fpg protein mechanism is an acidic group carrying out a similar function to Glu-23 in endonuclease V. Since Fpg normally carries out a β,δ-elimination reaction, it is highly unlikely that Fpg dissociates before δ-elimination. In fact, Fpg has been shown to be unable to promote δ-elimination in a DNA molecule already cleaved by a β-elimination event at an AP site (Bailly et al., 1989b). δ-Elimination has not been reported for endonuclease III or the *M. luteus* UV endonuclease. The absence of δ-elimination catalysis, even at high enzyme concentrations, could indicate that these proteins have an active site that will not easily accommodate the cleaved substrate, that the acidic group necessary for removal of the 2′-H in the β-elimination reaction is not properly situated to also remove the 4′-H for δ-elimination, or that these enzymes have even less affinity for their cleaved substrates than endonuclease V.

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