Evidence for an Imino Intermediate in the T4 Endonuclease V Reaction†

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ABSTRACT: Reductive methylation and site-directed mutagenesis experiments have implicated the N-terminal α-amino group of T4 endonuclease V in the glycosylase and abasic lyase activities of the enzyme. NMR studies have confirmed the involvement of the N-terminal α-amino group in the inhibition of enzyme activity by reductive methylation. A mechanism accounting for these results predicts that a (imino) covalent enzyme–substrate intermediate is formed between the protein N-terminal α-amino group and C_1' of the 5′-deoxyribose of the pyrimidine dimer substrate subsequent to (or concomitantly with) the glycosylase step. Experiments to verify the existence of this intermediate indicated that enzyme inhibition by cyanide was substrate-dependent, a result classically interpreted to imply an imino reaction intermediate. In addition, sodium borohydride reduction of the intermediate formed a stable dead-end enzyme–substrate product. This product was formed whether ultraviolet light-irradiated high molecular weight DNA or duplex oligonucleotides containing a defined thymine–thymine cyclobutane dimer were used as substrate. The duplex oligonucleotide substrates demonstrated a well-defined gel shift. This will facilitate high-resolution footprinting of the enzyme on the DNA substrate.

The enzyme T4 endonuclease V initiates the repair of UV-induced pyrimidine dimers in DNA. Chemical modification and site-directed mutagenesis studies of the N-terminal region of this enzyme have shown that the α-amino group of the enzyme is likely to be involved in the mechanism of catalysis (Schrock & Lloyd, 1991, 1993). These investigations demonstrated that one methyl group introduced into the enzyme molecule by reductive methylation was sufficient to inhibit both the glycosylase and abasic lyase activities of the enzyme, but that pyrimidine dimer-specific binding of the enzyme to the irradiated substrate was unaltered. At higher levels of modification (4.4 methyl groups per enzyme molecule) the enzyme did not bind to the irrradiated substrate. Under the salt conditions used, neither modified nor unmodified enzyme bound to unirradiated DNA. The α-amino group of the enzyme was shown to be the likely site of preferential methylation. Site-directed mutagenesis studies demonstrated that the identity of the N-terminal amino acid was unimportant as long as the α-amino group was a primary amine and was unaltered in its spatial relationship to the rest of the enzyme. A mechanism was proposed to account for these results, and the hallmark of this mechanism is an imino enzyme–substrate DNA intermediate (Figure 1). In the work described in this paper additional evidence for the involvement of the N-terminal α-amino group in this imino intermediate is reported and predictions arising out of this mechanistic proposal are tested.

Substrate-dependent inhibition by cyanide is characteristic of enzymes involving an obligatory imine intermediate in the catalytic reaction scheme (Cash and Wilson, 1966; Dixon and Webb, 1964). Pyridoxal phosphate-dependent enzymes and decarboxylases are some of the enzymes characterized by such mechanisms. If the endonuclease V reaction goes through this putative imino intermediate, then the enzyme should be inhibited by CN^- in a substrate-dependent manner. Seawell et al. (1980), while developing a filter binding assay for endonuclease V, observed that preformed enzyme-irradiated DNA complexes were stabilized by NaCN. In addition to inhibition by NaCN, the putative imino intermediate should be converted into a covalent enzyme–substrate complex by reducing agents such as $NaBH_4$.

Mechanistic proposals sharing some common features with Figure 1 have been advanced for other enzymes that carry out analogous reactions at the sites of other types of damaged bases in DNA or that convert nucleotides to free bases and sugar phosphates. Kow and Wallace (1987) predicted that an essential amino group was present in the active site of *Escherichia coli* endonuclease III, an enzyme that carries out sequential *N*-glycosylase and abasic lyase reactions at thymine glycol sites in DNA. These sequential reactions are similar to those catalyzed by endonuclease V, and these two enzymes follow identical stereochemical courses during cleavage at an abasic site (Mazumder et al., 1991), even though the primary and tertiary structures are not similar. Kow and Wallace also predicted that a stable enzyme–DNA imino intermediate was formed in the endonuclease III reaction. Mentch et al. (1987) determined the transition-state structure for the AMP (adenosine monophosphate) *N*-glycosidase reaction catalyzed by AMP nuclosidase, a reaction similar to the glycosylase step catalyzed by endonuclease V. Mentch et al. found the mechanism that best explained their kinetic isotope effects was one that proceeded by way of an early S_N1 transition state with significant residual bond order in the glycosidic bond and protonation of the adenine base. The enzyme was found to enforce weak bonding of an enzyme-bound water molecule to C1′ in the transition state. We propose that the N-terminal α-amino group of endonuclease V is the attacking nucleophile in a role similar to that played by the enzyme-bound water molecule in the AMP nucleosidase mechanism.

EXPERIMENTAL PROCEDURES

Enzyme and DNA Preparations. Highly purified endonuclease V was prepared from an overproducing clone of the *den*V gene as previously described (Prince et al., 1991).

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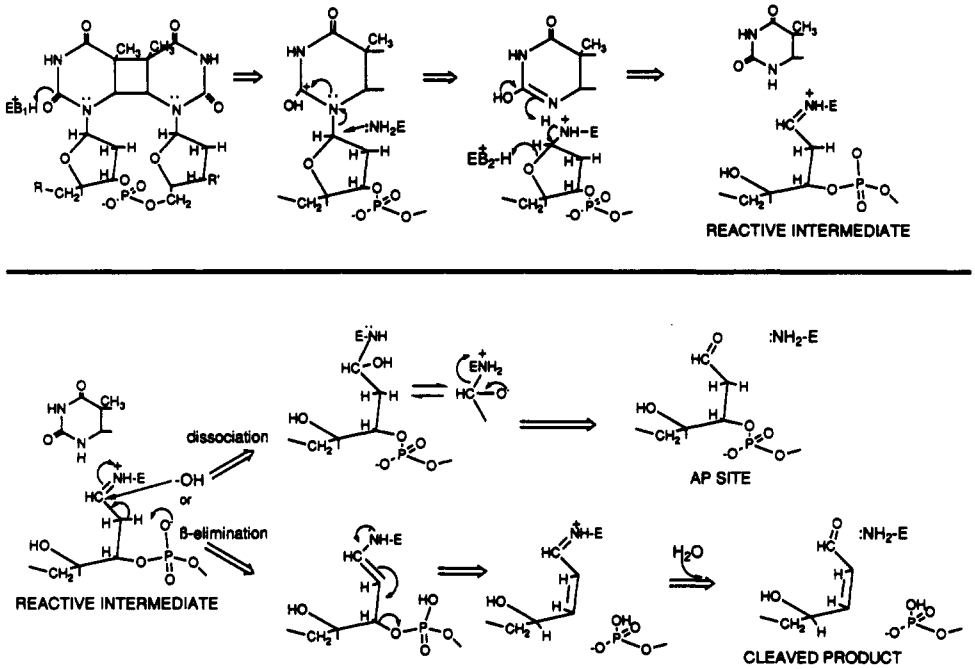

FIGURE 1: Reaction scheme proposed for endonuclease V.

An oligonucleotide containing a specific *cis-syn* pyrimidine dimer and its complementary oligonucleotide were the generous gifts of Colin Smith and John-Stephen Taylor, Washington University, St. Louis, MO. They were prepared as described in Taylor et al. (1990). The *cis-syn* dimer oligonucleotide sequence was:

5′-AGCTACCATGCCTGCACGAATTAAGCAATT-
CGTAATCATGGTCATAGCT-3′

The underlined positions indicate the thymines in the dimer.

Substrate DNA for enzyme reactions was prepared as follows. Plasmid pBR322 DNA, at concentrations of 0.5–1.25 $\mu g/\mu L$, was irradiated with constant stirring using two 15-W germicidal lamps at a distance of approximately 1 m. UV fluences used ranged between 230 and 1000 J/m^2 and are specified in the individual figure legends. All UV irradiations resulted in greater than 15 pyrimidine dimers per plasmid DNA molecule.

^{13}C-NMR of Endonuclease V. Endonuclease V was reductively methylated in the presence of $H^{13}CHO$ and $H^{14}CHO$. Reductive methylation reactions were carried out in 50 mM $Na_{1.5}H_{1.5}PO_4$ (pH 6.8) buffer as previously described (Schrock & Lloyd, 1991), except that a mixture of $H^{13}CHO$ and $H^{14}CHO$ was used. Two preparations of $^{13,14}CH_3$-endonuclease V were made: one with 1 and the other with 3.5 modifications per endonuclease V molecule. Enzyme was denatured by addition of SDS to 3% final concentration and heating to 95 °C. Titrations of the enzyme solutions were carried out by the addition of small quantities of 5 N NaOH. The pH of each solution was measured before and after each spectrum was taken. The ^{13}C NMR spectra were taken using Bruker narrow bore AM 200- and 400-mHz spectrometers.

Endonuclease V Reactions. For demonstration of the covalent attachment of endonuclease V to substrate DNA in $NaBH_4$-treated reaction mixtures, commercial calf thymus DNA from Sigma was further purified in a CsCl–ethidium bromide density gradient. After removal of the ethidium bromide and CsCl, the DNA was irradiated at 0.1 $\mu g/\mu L$ for a total fluence of 1100 J/m^2. This DNA preparation (10 mL) was reacted for 2 h at 37 °C in the presence of 0.2 M $NaBH_4$ with an amount of endonuclease V (100 μg) sufficient to yield a ratio of approximately one endonuclease V molecule per 20 pyrimidine dimers. This ratio was chosen such that essentially all the active endonuclease V molecules would be trapped even if the trapping conditions were only 5% efficient. The reaction was terminated by freezing the mixture.

Endonuclease V was shown to be covalently attached to the irradiated DNA by separation of the mixture on a Superose 12 FPLC gel filtration column of 1.7-cm diameter by 29.5-cm length in 10 mM Tris, pH 7.6, 100 mM NaCl. Endonuclease V was small enough to be included in the column separation volume, whereas the calf thymus DNA was sufficiently large to be excluded from the column matrix (Nickell & Lloyd, 1991). The DNA in the effluent fractions was quantitated by absorbance at 260 nm, and endonuclease V was quantitated by dot immunoblot as follows: Fractions of approximately 0.5 mL were collected, and the absorbance at 260 nm was measured. After the fractions were adjusted to 10 mM $CaCl_2$, they were treated with 1 μg per tube of micrococcal nuclease at 37 °C for 2 h. The fractions were individually filtered through a nitrocellulose filter in a 96-well dot blot apparatus. After the filter was treated for 2 h with 3% gelatin in Dulbecco's phosphate buffered saline (PBS) to block nonspecific binding, it was incubated overnight in 3% gelatin in PBS containing a monoclonal antibody to endonuclease V. The next day the filter was washed three times (room temperature, 20 min per wash) with PBS and incubated for 4 h at room temperature with 1% gelatin containing a secondary antibody (rabbit anti-mouse immunoglobulin) coupled to horse radish peroxidase. After being washed three times as described above, the dot blots on the filter were developed with 4-chloro-1-naphthol and H_2O_2: 32 mg of 4-chloro-1-naphthol was dissolved in 12 mL of methanol. This was added to 60 mL of 0.02 M Tris, pH 8.0, 0.5 M NaCl. A 120-μL portion of 30% H_2O_2 was added, and the filter was incubated in this mixture until the formation of the colored peroxidase product on those blots containing endonuclease V. The filter was throughly washed with distilled water and dried, and the peroxidase product was quantitated by scanning the filter with a gray-scale scanner and measuring the intensity of the dots using the NIH Image program. NIH Image is a public domain image processing and analysis package written by Wayne Rasband for the Apple

Macintosh. It can be obtained by anonymous file transfer from zippy.nimh.nih.gov [Internet address 128.231.98.32].

The identification of the site of attachment of the enzyme to DNA was carried out as follows: The preparation of endonuclease V covalently attached to UV-irradiated calf thymus DNA described above (100 μL) or a highly purified preparation of endonuclease V (100 μL) was diluted with 400 μL of 88% formic acid. These preparations were subdivided into two 250-μL aliquots. To one of the aliquots was added 250 μL of 70% formic acid containing 100 mg/mL of cyanogen bromide (Sigma). To the other aliquot was added 250 μL of 70% formic acid without cyanogen bromide. Each of these four preparations was incubated at room temperature for approximately 18 h. The preparations were dried in a vacuum centrifuge, resuspended in 500 μL of 20% trichloroacetic acid with vigorous agitation, and left on ice for 45 min. After centrifugation at 4 °C for 15 min at top speed in an Eppendorf microcentrifuge, the supernatent fluid was carefully removed, and the pellets were dried in a vacuum centrifuge. The dried pellets were resuspended in 20 μL of SDS boiling buffer (30% glycerol, 3% sodium dodecyl sulfate, 15% 2-mercaptoethanol, 0.02% bromphenol blue, 250 mM Tris–HCl, pH 7). The samples were neutralized with the smallest possible volume of 4 M Tris–HCl, pH 8. Each sample was boiled for 3 min and separated on an 8–25% gradient SDS gel in a Pharmacia PhastSystem at 15 °C for a total of 65 V-h at a maximum of 10 mA per gel. Markers used were: ovalbumin (46 kDa, KDa), carbonic anhydrase (30 KDa), trypsin inhibitor (21.5 KDa), lysozyme (14.3 KDa), aprotinin (6.5 KDa), insulin B chain (3.4 KDa), and insulin A chain (2.3 KDa). The gel was stained overnight in 0.05% Coomassie Brilliant Blue in destain (6:3:1 by volume of water–methanol–glacial acetic acid). Subsequently, the gels were exhaustively destained in this mixture. Under these conditions all peptides smaller than lysozyme were lost from the gel.

Unless otherwise indicated in the figure legends, all enzyme assays were done in reactions containing 0.05 μg/μL of substrate DNA, 200 mM Na^+ (contributed from NaCl, NaCN, or $NaBH_4$ or mixtures of NaCl with one of the other two salts), and 25 mM $Na_{1.5}H_{1.5}PO_4$ (an equimolar mixture of NaH_2PO_4 and Na_2HPO_4, pH 6.8). Enzyme was diluted into 1 mg/mL bovine serum albumin, 25 mM $Na_{1.5}H_{1.5}PO_4$. For enzyme assays using circular plasmid DNA substrates, the amount of enzyme was selected such that residual unreacted form I DNA was still remaining at all time points. This maximized the portion of the reaction within the initial linear portion of the kinetic curve.

Analytical Methods. Forms I and II DNA (covalently closed circular and nicked circular plasmid DNA, respectively) were electrophoretically separated in 1% agarose gels in 1×TAE (40 mM Tris–acetate, pH 8.0, 1 mM EDTA). After electrophoresis the gels were stained overnight in 0.2 μg/mL of ethidium bromide in 1×TAE. The fluorescence intensities of gel bands corresponding to forms I and II of the plasmid DNA substrates were recorded on Polaroid 55 positive/negative 4 × 5 instant sheet film. White light transmission images of the negatives were captured as tagged image file format (TIFF) files using a charge-coupled device camera as part of the Imager system (American Synthesis Inc., 1177-C Quarry Lane, Pleasanton, CA 94566). The TIFF files were converted from transmission to absorbance mode, and the integrated absorbancies of the gel band images (proportional to fluorescence intensities in the original gel) were measured using Millipore BioImage Visage software on a Sun Sparc Station II workstation (BioImage Products, 777 E. Eisenhower Parkway, Suite 950, Ann Arbor, MI 48108). The raw integrated absorbancies of the bands corresponding to form I DNAs were multiplied by 1.42 to correct for the lowered binding of ethidium bromide to supercoiled DNA relative to the topologically relaxed forms (Lloyd et al., 1978). We have previously shown that endonuclease V, in high salt conditions (200 mM Na^+), randomly introduces single strand breaks at pyrimidine dimers in a population of substrate DNA molecules (Gruskin & Lloyd, 1986). For circular substrates, the assay method used cannot measure all single strand breakage events, only the first event on a form I substrate. Since each molecule contains many pyrimidine dimers as potential single strand breakage sites and breakage is randomly distributed over the population of substrate molecules under the conditions used, the proportion of residual form I DNA molecules during the reaction is given by the zero term of the Poisson distribution, such that:

$$I_t = I_0 \exp(-\lambda_t)$$

where I_t is the mass fraction of form I DNA molecules at reaction time t, I_0 is the fraction of form I DNA molecules at time 0, and λ_t is the average number of endonuclease V-catalyzed single strand breaks per DNA molecule at reaction time t. Let r be the steady state reaction rate for single strand breakage, expressed on a per DNA molecule basis. If the rate is constant over the course of the reaction, $\lambda_t = rt$, then

$$-\ln I_t = -\ln I_0 + rt$$

A plot of the negative logarithm of the fraction of (unbroken) form I DNA molecules remaining versus time is a straight line with slope r.

Reaction of endonuclease V with a duplex oligonucleotide containing a specific *cis-syn* pyrimidine dimer was performed as follows: The 49-mer containing a *cis-syn* thymine dimer described above was 5′-end labeled with ^{32}P using polynucleotide kinase and γ-^{32}P-ATP. The kinase reaction was terminated by heating to 65 °C for 5 min. An equimolar concentration of the complementary oligonucleotide and a large excess of unlabeled ATP was added. The resulting labeled duplex oligonucleotide was incubated with various amounts of endonuclease V (60, 6, or 0.6 ng per reaction) in 25 mM $Na_{1.5}H_{1.5}PO_4$, pH 6.8, 100 mM KCl, 100 μg/mL of bovine serum albumin, 1 mM EDTA plus additions of 100 mM NaCl, NaCN, or $NaBH_4$. The reaction products were subjected to electrophoresis through a 6% nondenaturing polyacrylamide gel.

RESULTS

Demonstration That Methylation of the N-Terminal α-Amino Group Inhibits the Endonuclease V Reaction. Evidence that the N-terminal α-amino group is preferentially methylated over the lysine ε-amino groups of endonuclease V was obtained from ^{13}C-NMR spectra of $^{13,14}CH_3$-endonuclease V. Jentoft and Dearborn (1979) and Jentoft et al. (1981) have demonstrated that resonances of the α-[^{13}C](methylamino) and ε-[^{13}C](methylamino) groups of ribonuclease A differ by 2–3 ppm. They also demonstrated that the pK_a of the amino group could be measured by chemical shift differences as a function of pH. This approach was used to identify the preferentially methylated amino group of endonuclease V. Two preparations of $^{13,14}CH_3$-endonuclease V were made: one had 1 and the other had 3.5 $^{13,14}CH_3$-groups per endonuclease V molecule. The neutral pH ^{13}C-NMR spec-

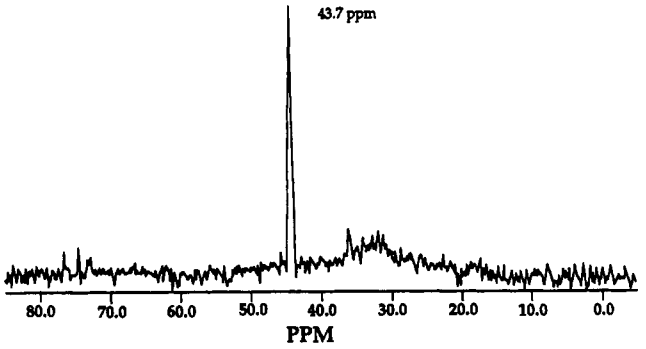

FIGURE 2: ^{13}C NMR spectrum of native endonuclease V modified to 1 $^{13}CH_3$ groups per enzyme molecule. Endonuclease V (1 mg/mL) was [^{13}C]methylated as described in the Experimental Procedures section. The ^{13}C NMR spectrum of the enzyme at neutral pH was obtained by scanning for 16 h at 12 °C on a Bruker narrow bore AM 400-MHz NMR spectrometer.

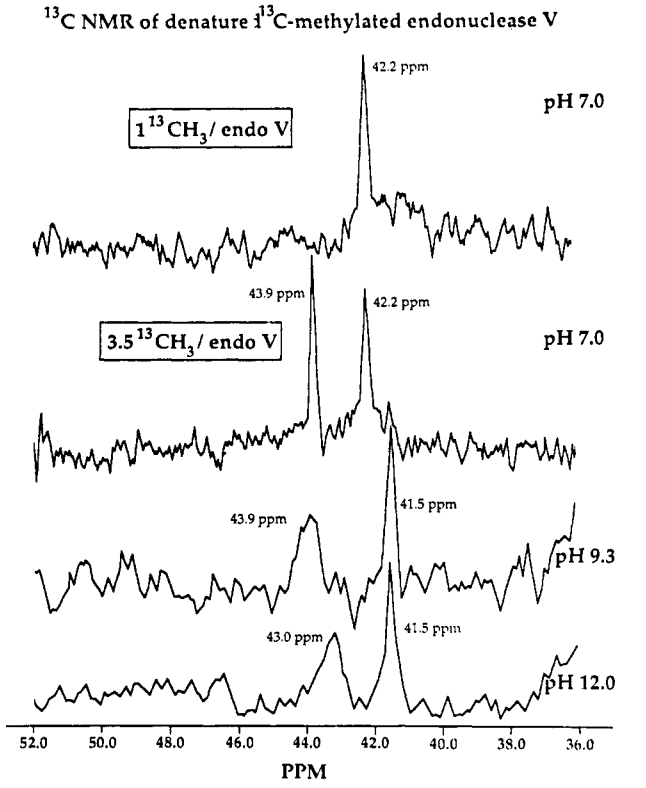

FIGURE 3: ^{13}C NMR spectra of 3% SDS-denatured endonuclease V modified to 1 $^{13}CH_3$ group (upper panel) and 3.5 $^{13}CH_3$ groups (other panels) per enzyme molecule. Endonuclease V (1 mg/mL) was [^{13}C]methylated and denatured as described in the Experimental Procedures section. The ^{13}C NMR spectrum of the enzyme in 3% SDS and at neutral pH was obtained by scanning for 16 h at 12 °C on a Bruker narrow bore AM 200-MHz NMR spectrometer. The pH was as indicated in the panels. Adjustment of pH was done by addition of a small amount of 5 N HaOH.

trum of the native 1 modification per molecule preparation had a single resonance at 43.7 ppm (Figure 2). This indicated that a single residue was preferentially methylated at early times in the methylation reaction. This preparation was also denatured with 3% SDS so that the effects of the local protein environment on the pK_as (and, therefore, the chemical shifts) of the α- or ϵ-(methylamino) groups were eliminated. The denatured preparation also gave a single resonance, but it was shifted to approximately 42.2 ppm (Figure 3, panel described as 1 CH_3/endo V, pH 7.0). The neutral pH ^{13}C-NMR spectrum of the SDS-denatured 3.5 modifications per molecule preparation, however, showed two resonances at 43.9 and 42.2

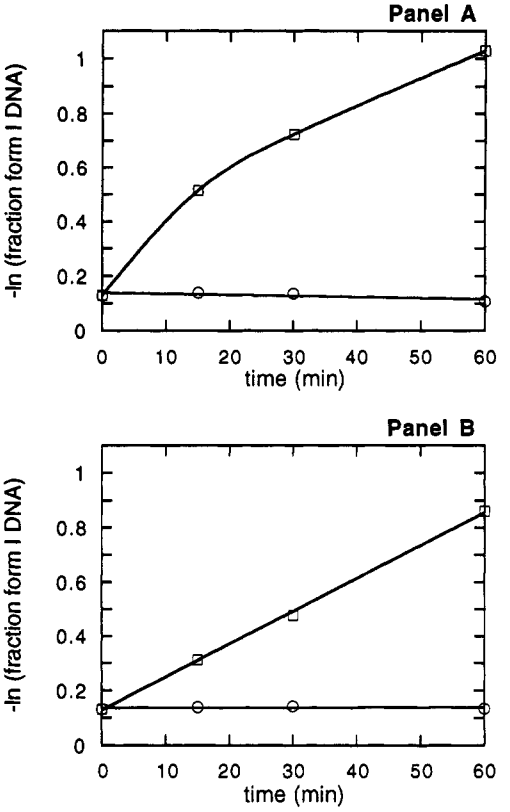

FIGURE 4: Panel A. Endonuclease V was preincubated for 1 h with 0.2 M NaCl (open squares) or NaCN (open circles). After 1000-fold dilution, the enzyme was reacted with pBR322 DNA (irradiated with 1000 J/m^2 at 0.8 μg/mL) as substrate in a reaction mixture containing 0.2 M of the same salt used in the preincubation. Panel B is the same except that the preincubation was with 0.2 M NaCN in both experimental series and the reactions were carried out in the presence of 0.2 M of NaCl (open squares) or NaCN (open circles).

ppm, presumably from the ϵ- and α-(methylamino) groups, respectively (Figure 3, panel described as 3.5 CH_3/endo V, pH 7.0). The identity of each resonance was confirmed by repeating the experiment at pH 9.3 and 12 (Figure 4, lower two panels). The upfield resonance shifted from 42.2 to 41.5 ppm as the pH was raised from 7.0 to 9.3 but did not shift further between pH 9.3 and 12. The downfield resonance did not shift between pH 7.0 and 9.3 but changed from 43.9 to 43.0 ppm as the pH was changed from 9.3 to 12. These results confirm that the upfield resonance originates from a group with the pK_a of an α-(methylamino) group, and the downfield resonance originates from an ϵ-(methylamino) group. The resonance originating from the α-(methylamino) group was the only one present in the spectrum of the singly methylated enzyme. These results indicate that the endonuclease V N-terminal α-amino group is preferentially methylated over the lysine ϵ-amino groups of the enzyme.

Cyanide Inhibition Is Substrate Dependent. In data not shown the dependence of reaction kinetics on the concentration of NaCN in the reaction mixture was measured at constant total Na^+ concentration (0.2 M). The estimated concentration range resulting in 50% inhibition was 3–5 mM NaCN.

Figure 4, panels A and B, show the results of kinetic experiments demonstrating that cyanide inhibits the endonuclease V reaction in a substrate-dependent manner. Panel A shows the kinetics of reactions catalyzed by endonuclease V which had been preincubated for 1 h (in the absence of substrate) in 0.2 M NaCN (open circles) or NaCl (open squares). After 1000-fold dilution, the enzyme assays were performed with irradiated plasmid DNA substrates in the

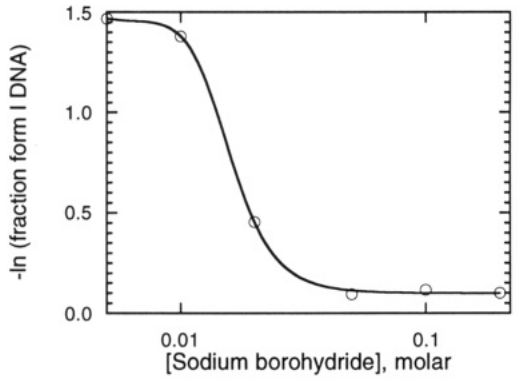

FIGURE 5: Endonuclease V reactions were performed in the presence of the indicated concentration of $NaBH_4$. NaCl was added to make the salt concentration 0.2 M in all reactions. Substrate was pBR322 DNA which had been irradiated with 235 J/m^2 at 0.5 μg/mL. There was no preincubation step.

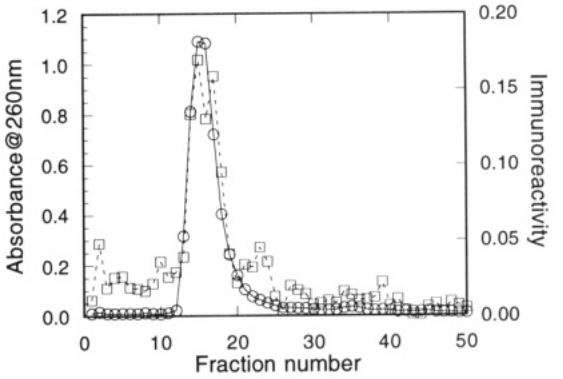

FIGURE 6: $NaBH_4$-reduced reaction mixtures were analyzed by FPLC gel filtration chromatography as described in the Experimental Procedures section. The absorbance at 260 nm is plotted as the open circles, and the relative immunoreactivity as determined by the methods described is plotted as the open squares. The position of the 260 absorbance peak coincides with the void volume of the column. Under these conditions, uncomplexed endonuclease V is included in the column volume.

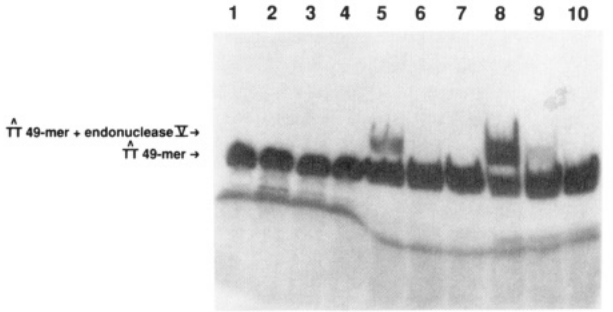

FIGURE 7: 49-mer oligonucleotide containing the specific *cis-syn* thymine dimer was reacted with endonuclease V and the mixture separated by nondenaturing 6% polyacrylamide gel electrophoresis as described in the Experimental Procedures section. Lane 1 is the 49-mer alone. Lanes 2–4 resulted from reactions containing decreasing concentrations of endonuclease V in 0.2 M NaCl. Lanes 5–7 resulted from reactions containing decreasing concentrations of endonuclease V in 0.2 M NaCN instead of NaCl. Lanes 8–10 are similar except that the reactions contained $NaBH_4$.

presence of the same salt used in the preincubation. These results show that preincubation with NaCl does not inhibit enzyme activity but that NaCN, present in both the preincubation and in the reaction mixture, completely inhibits activity. Panel B shows enzyme kinetics after 1 h of preincubation with NaCN. After preincubation, the enzyme preparations were diluted 1000-fold and assayed in the presence of NaCN (open circles) or NaCl (open squares). The preincubation with NaCN in the absence of substrate did not prevent enzyme activity when the assay was performed in NaCl solutions of equal Na^+ concentrations to that present in the preincubations with NaCN.

Inhibition by $NaBH_4$ and Crosslinking of Substrate to Enzyme. CN^- inhibition of the enzyme reaction in a substrate-dependent manner as demonstrated above is characteristic of a mechanism involving an imino enzyme–substrate intermediate. That the imino intermediate can be reduced to a stable covalent enzyme–substrate complex is another hallmark of this mechanism. Figure 5 shows the concentration dependence of the inhibition of the reaction by $NaBH_4$ (inhibition of the reaction would be observed since an obligatory intermediate is being converted to a nonreactive form). Similar results were obtained using $NaCNBH_3$ as the reducing agent (data not shown).

In order to determine whether a stable covalent enzyme–DNA product was formed by $NaBH_4$ reduction of a putative imino reaction intermediate, highly purified (CsCl-banded) calf thymus DNA was irradiated and used as substrate in an endonuclease V reaction containing 0.2 M $NaBH_4$. After 2 h the DNA was isolated and separated from uncomplexed enzyme on an FPLC gel filtration column. The enzyme in the column effluent was quantitated by an immuno-dot blot procedure as described in Experimental Procedures. Figure 6 shows that the majority of the enzyme coeluted with the DNA in the void volume of the column.

A stable covalent intermediate was also obtained when a duplex 49-mer oligonucleotide containing a site-specific *cis-syn* pyrimidine dimer was used as a substrate in reactions carried out in the presence of NaCN or $NaBH_4$. As shown in Figure 7, a stable complex between the enzyme and the defined substrate was formed when either NaCN or $NaBH_4$ was present in the reaction. The covalent enzyme–substrate complex migrated more slowly through the polyacrylamide gel than the DNA substrate alone. The amount of this complex increased as the amount of endonuclease V was increased.

Identification of the Amino Group Involved in Substrate–Enzyme Crosslinking. An experiment to identity the amino group involved in the covalent enzyme–substrate complex formed in the presence of $NaBH_4$ is shown in Figure 8. Panel A illustrates the outcome of this experiment under two different scenarios: (1) the amino group involved is the α-amino group (top diagram of panel A), and (2) the amino group involved is a lysine ϵ-amino group (lower diagram of panel A). The endonuclease V sequence contains 10 lysine residues, all C-terminal to the single methionine residue at position 18. If the N-terminal α-amino participates in the imino intermediate, cyanogen bromide cleavage of the product resulting from $NaBH_4$ trapping the intermediate will yield a 14 KDa free peptide, corresponding to the C-terminus of the enzyme, and a 1.9 KDa peptide still covalently attached to DNA. If a lysine ϵ-amino group is involved in the intermediate, the 14 KDa C-terminal peptide will remain attached to the DNA, and the 1.9 KDa N-terminal peptide will be released. Thus, the key point is whether or not the 14 KDa C-terminal peptide is released in free form. Panel B (lanes 4 and 5, counting from the left of the photograph) shows that a control treatment of pure endonuclease V with cyanogen bromide does form the expected 14 KDa peptide. (The cyanogen bromide reaction did not go to completion in this experiment.) CNBr treatment of the covalent DNA–endonuclease V product also releases a 14 KDa peptide (lanes 2 and 3), indicating that the N-terminal α-amino group is the one involved in the enzyme–substrate covalent complex.

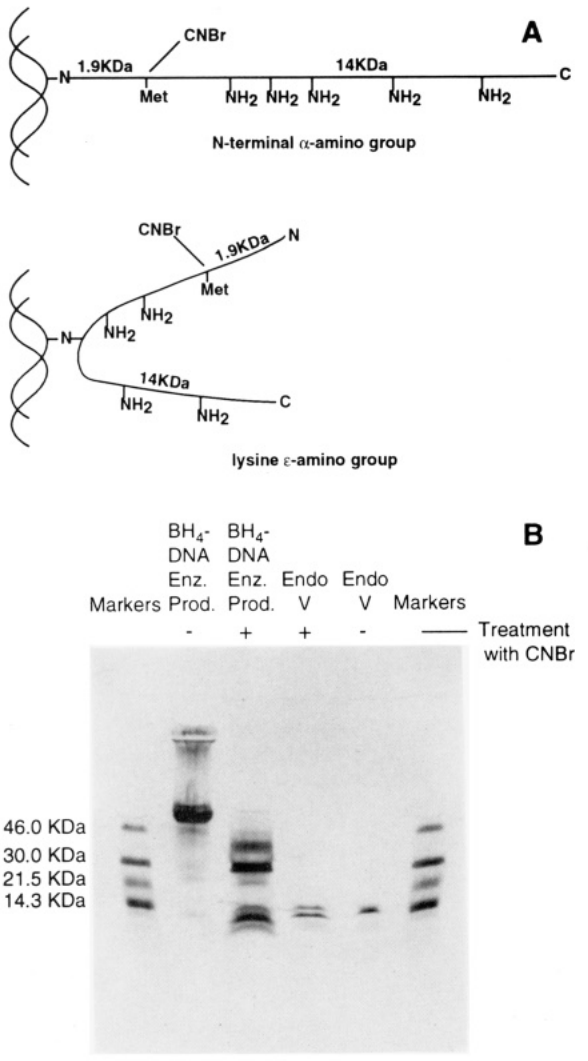

FIGURE 8: $NaBH_4$-reduced reaction product was treated with cyanogen bromide as described in the Experimental Procedures section. Panel A diagrams the scenarios for the two possible identities of the amino group involved in the reaction mechanism. Panel B shows the results of the cyanogen bromide treatment. The lanes are as follows (left to right): (1) protein size markers; (2) the $NaBH_4$-reduced enzyme–DNA product, treated with 70% formic acid, but not treated with cyanogen bromide; (3) the same as lane 2, but treated with cyanogen bromide; (4) highly purified endonuclease V treated with cyanogen bromide in 70% formic acid; (5) the same as lane 4, but not treated with cyanogen bromide; (6) protein size markers.

DISCUSSION

This laboratory previously demonstrated that reaction of endonuclease V with [^{14}C]formaldehyde in the presence of $NaCNBH_3$ resulted in 70–80% inhibition of enzyme activity when an average of 0.8 methyl groups had been introduced into the enzyme molecule. Radioactivity was found exclusively in a V8 protease peptide that could not be sequenced and that had the amino acid composition of the N-terminal peptide of the enzyme. This was taken as evidence for the unusual involvement of the α-amino group of the enzyme in the reaction scheme, and a mechanism incorporating these results was proposed.

The ^{13}C NMR spectra as a function of pH of two enzyme preparations modified to 1 and 3.5 methyl groups per enzyme molecule solidify the argument that the N-terminal α-amino group of endonuclease V was preferentially methylated over the lysine ϵ-amino groups of the enzyme. The pK_a of an α-amino group is approximately 8, and the pK_a of a lysine ϵ-amino group is approximately 10 under denaturing conditions (Stark, 1967; Tanford & Havenstein, 1956). Since methylation of a primary amino group does not dramatically alter its pK_a (Jentoft et al., 1979), the behavior of the two resonances as a function of pH in the methylated enzyme preparation containing 3.5 methyl groups per molecule allowed the upfield resonance to be assigned to the methylated N-terminal α-amino group. The downfield resonance was assigned to (a) methylated lysine ϵ-amine(s). Since the upfield resonance was the only one found early in the course of the methylation reaction, the N-terminal α-amino group must be the most reactive to methylation.

The mechanism proposed for endonuclease V has as one of its hallmarks the presence of an imino enzyme–substrate intermediate. It is well known that such intermediates are reactive with cyanide and are susceptible to reduction, forming stable covalent complexes (Dixon & Webb, 1964; Cash & Wilson, 1966). The results in this paper indicate that the endonuclease V reaction displays the characteristics of a reaction with an imino enzyme–substrate intermediate. This gives substantial support to the mechanism proposed by Schrock and Lloyd (1991) and diagrammed in Figure 1. In addition, the results described here demonstrate that the amino group involved in the imino intermediate is the N-terminal α-amino group of the enzyme.

Doi et al. (1992) have shown that glutamate-23 is a key residue for the pyrimidine dimer glycosylase activity of endonuclease V. Substitution by aspartic acid or glutamine abolished glycosylase activity completely. Hori et al. (1992) showed that glutamate-23 was important for the abasic lyase activity of endonuclease V but that it could be substituted by aspartic acid with retention of some activity (k_{cat}/K_m approximately 50-fold lower than wild type). Since an oligonucleotide containing an (aminoethyl)phosphonate at the thymine dimer site was cleaved by an excess of enzyme, they concluded that glutamate-23 was acting as a general base in the abstraction of the *pro-S* 2′-hydrogen (Mazumder et al., 1989). However, glutamate-23 was shown to be required for the glycosylase step by Doi et al., so that, if it is involved in the abstraction of the *pro-S* 2′-hydrogen, glutamate-23 must play an additional role as well.

Inspection of the reaction mechanism of Figure 1 shows that the results of this paper are not at variance with the results of Doi et al. (1992) and Hori et al. (1992). The mechanism of Figure 1 explicitly involves a protonated imino intermediate that is likely to require (an) acidic group(s) in the active site to stabilize its charge. This role could be played by glutamate-23. Resolution of the roles of various amino acid residues in the endonuclease V reaction mechanism must await determination of a structure for the bound substrate–enzyme complex (perhaps stabilized by reduction as described herein). In a paper describing the crystal structure of the mechanistically related enzyme endonuclease III, Kuo et al. (1992) have implicated glutamate-112 and lysine-120 as the acidic and basic groups involved in a reaction scheme similar to Figure 1.

Rapid quench kinetic analyses of the enzyme reaction have been initiated to clarify the reaction details. Measurement of kinetic isotope effects by these fine structure kinetic methods may also allow a distinction between S_N1 and S_N2 mechanisms for the initial nucleophilic attack proposed in the overall enzyme reaction scheme of Figure 1.

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