Stereochemical Studies of the β-Elimination Reactions at Aldehydic Abasic Sites in DNA: Endonuclease III from *Escherichia coli*, Sodium Hydroxide, and Lys-Trp-Lys†

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ABSTRACT: The DNA strand cleavage reaction catalyzed by endonuclease III from *Escherichia coli* (endo III) on the 3′-side of aldehyde abasic sites proceeds by a syn β-elimination involving abstraction of the *2′-pro-S* proton and formation of a trans α,β-unsaturated aldose product; we previously reported the same stereochemical course for the reaction catalyzed by UV endonuclease V from bacteriophage T_4 (UV endo V) [Mazumder, A., Gerlt, J. A., Rabow, L., Absalon, M. J., Stubbe, J., & Bolton, P. H. (1989) *J. Am. Chem. Soc. 111*, 8029–8030]. Since the UV endo V does not contain an 4Fe–4S center, the 4Fe–4S center present in endo III need not be assigned a unique role in the β-elimination reaction. The β-elimination reactions that occur under alkaline conditions (0.1 N NaOH) and in the presence of the tripeptide Lys-Trp-Lys proceed by anti β-elimination mechanisms involving abstraction of the *2′-pro-R* proton and formation of a trans α,β-unsaturated aldose product. The different stereochemical outcomes of the enzymatic and nonenzymatic β-elimination reactions support the hypothesis that the enzyme-catalyzed reactions may involve general-base-catalyzed abstraction of the *2′-pro-S* proton by the internucleotidic phosphodiester leaving group.

In *Escherichia coli* excision of damaged bases from DNA occurs by either of two pathways, depending upon the identity of the damaged base [for a recent review, see Myles and Sancar (1989)]. Both pathways involve excision of the damaged base by a glycosylase to yield an aldehydic abasic site. As an example of the first pathway, uracil-DNA N-glycosylase (UraGly) catalyzes hydrolysis of the *N*-glycosyl bond to uracil, a damaged base. The abasic sites produced by UraGly are excised by other enzymes as deoxyribose 5-phosphate, and the resulting gap is repaired. A second pathway involves bifunctional enzymes that excise the damaged base and then catalyze strand cleavage on the 3′-side of the abasic site. In the case of pyrimidine photodimers, UV endonuclease V from bacteriophage T_4 (UV endo V) cleaves the 5′-glycosyl bond to a photodimer to release an abasic site as a free intermediate (Nakabeppu et al., 1982); the strand cleavage occurs by a β-elimination mechanism (Mazumder et al., 1989). In the case of damaged pyrimidines, endonuclease III from *E. coli* (endo III) cleaves the *N*-glycosyl bond to the damaged base and, without releasing an aldehydic abasic site as a free intermediate (Kow & Wallace, 1987), cleaves the strand by a β-elimination mechanism as described in this article. Endo III also catalyzes strand cleavage at preexisting abasic sites. The α,β-unsaturated aldose 5-phosphate generated at the 3′-end of DNA is excised by other enzymes, and the resulting gap is repaired.

Despite the importance of aldehydic abasic sites as intermediates in the base excision repair pathways, little is known about their structures and reactivities in solution. Recently, both ^{13}C NMR and ^{17}O NMR spectroscopies have been used to establish that the predominant form of the abasic site is a 40:60 mixture of α- and β-hemiacetals (Manoharan et al., 1988b) and that the ring-opened aldehyde tautomer, the putative reactive species in alkaline lability, represents less than 1% of the total abasic sites (Wilde et al., 1989).

Indirect evidence was reported recently by the laboratories of Verly (Bailly & Verly, 1987; Bailly et al., 1989) and Linn (Kim & Linn, 1988) that the strand cleavage reactions catalyzed by UV endo V and by endo III proceed by a β-elimination rather than a hydrolysis mechanism. The first definitive evidence supporting the β-elimination mechanism was the observation that an abasic site labeled with ^{13}C in the 1′- and 3′-carbons is converted by UV endo V into an α,β-unsaturated aldehyde (Manoharan et al., 1988a). Subsequent studies defined the syn stereochemical course of this β-elimination reaction: the *2′-pro-S* hydrogen is abstracted and a trans α,β-unsaturated aldose is produced (Scheme I; Mazumder et al., 1989).

This article describes studies of the mechanism of analogous strand cleavage reaction catalyzed by endo III. Whereas the structure of the product of the reaction catalyzed by UV endo V was established initially by ^1H NMR spectroscopy (Mazumder et al., 1989), we now have developed HPLC methods that permit rapid identification of products of β-elimination reactions. With a variety of substrates, the reactions catalyzed

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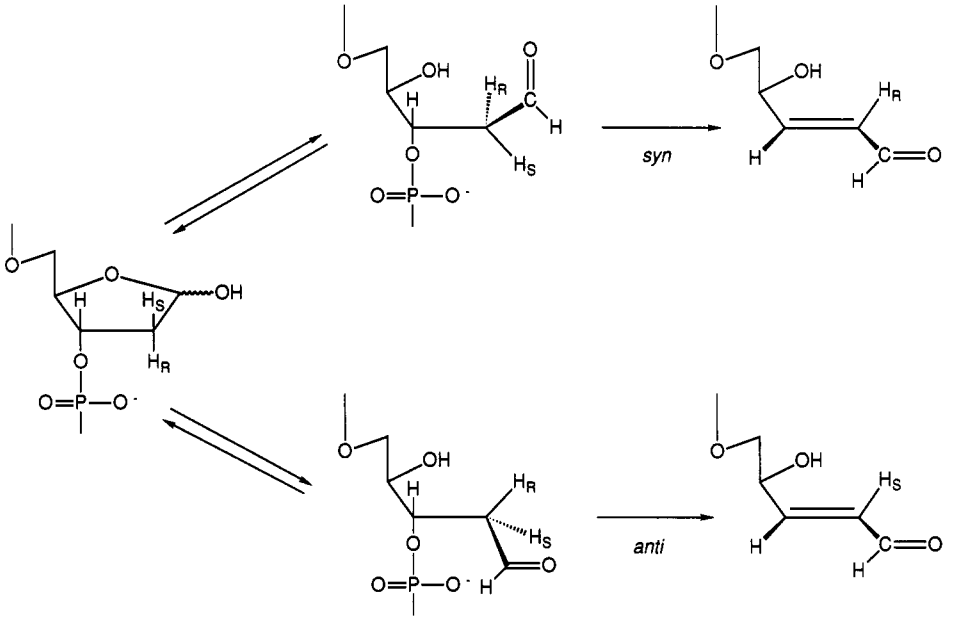
Scheme I



by UV endo V and endo III proceed by the same syn stereochemical course, thereby suggesting that the 4Fe–4S center present in endo III (Cunningham et al., 1989) is not uniquely necessary for catalysis of the β-elimination reaction.

We also report that the strand cleavage reactions at abasic sites that occur under nonenzymatic alkaline conditions or in the presence of the tripeptide Lys-Trp-Lys (Brun et al., 1975; Pierre & Laval, 1981) proceed by anti β-elimination mechanisms. The different stereochemical consequences observed in the enzymatic and nonenzymatic β-elimination reactions support the hypothesis that phosphodiester that is eliminated is the general base abstracting the 2′-*pro-S* proton in the enzymatic reactions.

Materials and Methods

Enzymes and Reagents. UraGly was purified to electrophoretic homogeneity as described by Lindahl et al., (1977) from *E. coli* strain N5219 transformed with pBD396; pBD396 was the gift of Dr. Bruce K. Duncan, Institute for Cancer Research, Philadelphia, PA. UraGly is stored at −20 °C in 30 mM HEPES–NaOH, pH 7.4, containing 5% glycerol, 2 mM dithiothreitol, and 1 mM EDTA. UV endo V was purified to electrophoretic homogeneity as described by Nakabeppu et al. (1982) from *E. coli* strain JM105 transformed with pTACdenV; pTACdenV was the gift of Dr. Errol C. Friedberg, Stanford University School of Medicine. UV endo V is stored at −20 °C in 10 mM potassium phosphate, pH 7, containing 10% ethylene glycol, 2 mM β-mercaptoethanol, and 2 mM EDTA. Endo III was purified as described by Asahara et al. (1989) from *E. coli* strain $\lambda N99_{cI857}$ transformed with pHIT1. Endo III is stored at −20 °C in 100 mM potassium phosphate, pH 6.6, containing 50% glycerol, 0.1 mM dithiothreitol, and 0.005% Triton X-100.

Calf intestine phosphatase and nuclease P_1 were obtained from Pharmacia. The Klenow fragment of DNA polymerase I from *E. coli* was obtained from Boehringer Mannheim Biochemicals. Polynucleotide kinase was obtained from New England Biolabs. The tripeptide Lys-Trp-Lys was obtained from Sigma. All other commercial reagents were of the best grade available.

The oligonucleotides $d(T_7UT_8)$, $d(A_{16})$, d(CGCAG), d(CGCAGUCAGCC), d(GGCTGACTGCG), and d(GGCTGACTGCGTTTT) were prepared with Biosearch 8750 or Microsyn 1450A automated DNA synthesizers. The concentrations of these oligonucleotides were estimated by assuming that a 50 μg/mL solution has an absorbance of 1.0 at 260 nm.

[2′-*proR*-^{3}H]dUTP was prepared by the reduction of UTP in ^3H_2O catalyzed by ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*; the specific radioactivity was 480 cpm/nmol. [2′-*pro-S*-^{3}H]dUTP was prepared by the enzymatic reduction of [2′-^{3}H]UTP in H_2O (Mazumder et al., 1989); the specific radioactivity was 2500 cpm/nmol.

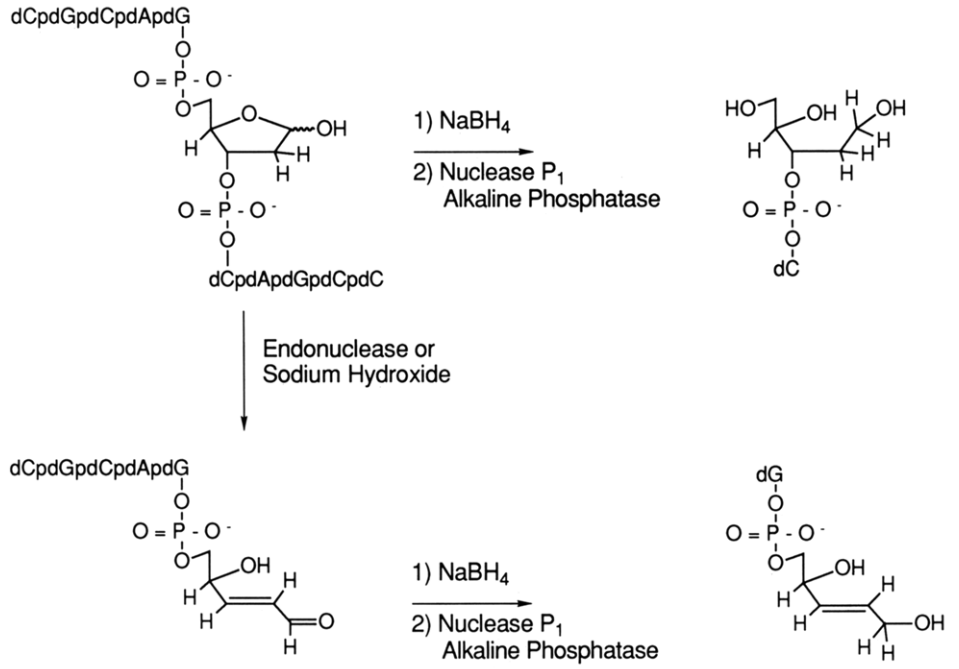
Heteroduplexes formed from d(CGCAGUCAGCC) base paired with d(GGCTGACTGCG) and labeled with ^{3}H in either the 2′-*pro-R* or 2′-*pro-S* position of the deoxyuridine residue were prepared by primer extension of d(CGCAG) annealed to d(GGCTGACTGCG) by the Klenow fragment for 12 h at 16 °C in the presence of the appropriate [^{3}H]dUTP, dATP, dCTP, and dGTP.

General Reaction and Analysis Conditions. Uracil was removed from both oligonucleotides and polynucleotides containing deoxyuridine residues by reaction in the presence of UraGly in 30 mM HEPES–NaH, pH 7.5, containing 50 mM NaCl and 1 mM EDTA. Sufficient enzyme, as assessed by small-scale reactions, was added to effect complete removal of uracil in 3 h at 37 °C. Tha abasic site so generated is subsequently designated D for *d*eoxyribose.

Endonuclease-catalyzed β-elimination reactions were performed by adding enzyme directly to the UraGly reaction mixture containing the in situ generated substrate with the abasic site(s). For those studies in which product analyses were conducted by HPLC, sufficient enzyme was added to effect complete reaction in times ranging from 60 to 180 min.

For analyses by HPLC, aliquots of enzyme-catalyzed β-elimination reactions (typically 40–120 μL) were quenched by cooling to 4 °C and addition of $NaBH_4$ to a final concentration of 0.1 M. After 30 min, the pH was adjusted to 5.5 with acetic acid, and $ZnCl_2$ was added to a final concentration of 1 mM; nuclease P_1 (5 units) was then added, and the reaction was allowed to proceed at 37 °C for 90 min. The pH was adjusted to 8.0 with NH_4OH, and $MgCl_2$ was added to a final concentration of 10 mM; calf intestinal phosphatase (5 units) was added, and the reaction was allowed to proceed at 37 °C for 30 min. Scheme II summarizes the analysis and shows the structures of the nucleotide esters derived from

Scheme II



unreacted abasic sites and β-elimination products. The reaction mixture was chromatographed at a flow rate of 1 mL/min on a C_{18} reverse-phase column (Beckman) by using a Beckman HPLC. The column was equilibrated in 5 mM ammonium acetate, pH 3.5 (solvent A), and methanol (solvent B) was used to elute the reaction components. Immediately following injection of the sample, the eluant was linearly changed from 100% solvent A to 88% solvent A over a period of 15 min. After an additional 10 min, the eluant was linearly changed from 88% solvent A to 65% solvent A over a period of 5 min and then returned to 100% solvent A over a period of 2 min. Reduced abasic site (16 min when esterified to 3′-dCMP) and reduced β-elimination product (18.5 min when esterified to 5′-dGMP) were separately collected and quantitated by UV spectroscopy; radioactivity was quantitated with a Beckman LS7000 liquid scintillation counter. Reduced abasic site esterified to 5′-dCMP or elimination product esterified to 3′-dGMP were assumed to have extinction coefficients of 7 and 13.1 mM^{-1} cm^{-1}, respectively, at 260 nm.

Nonenzymatic reactions conducted under alkaline conditions were performed at 4 °C following addition of an equal volume of 0.2 N NaOH to a chilled solution of the oligonucleotide containing the in situ generated abasic site (pH 13). The reactions were quenched by the addition of HCl to a final concentration of 100 mM (pH 8).

Nonenzymatic reactions conducted in the presence of Lys-Trp-Lys were performed in 30 mM HEPES–NaOH, pH 7.5, containing 1 mM EDTA; NaCl was omitted from this buffer since this inhibits the strand cleavage reaction. Lys-Trp-Lys was added to a final concentration of 0.5 mM.

In control experiments, we have determined with ^3H-labeled substrates that neither excision of uracil by UraGly nor incubation of labeled abasic sites in buffer at pH 7 and 37 °C (in the absence of either UV endo V or endo III) is accompanied by detectable β-elimination or labilization of ^3H.

Preparation of an Abasic Site in d(T_7UT_8). d(T_7UT_8) (125 μg) was 5′-end labeled and purified by polyacrylamide gel electrophoresis (TBE buffer) in the presence of 7 M urea; the labeled 16-mer was eluted from the gel and purified by anion-exchange chromatography (NACS column) and gel filtration (NAP-25 column). The uracil was excised with 100 μg of UraGly to yield d(T_7DT_8).

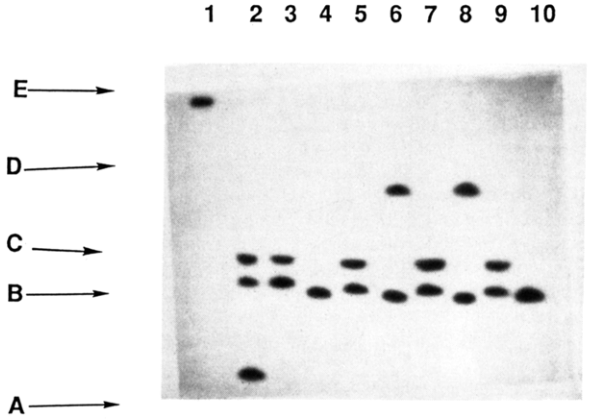
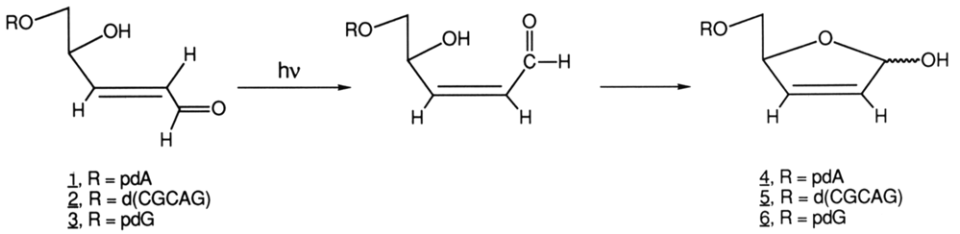


FIGURE 1: Electrophoresis of the reaction product obtained from [^{32}P]pdT_8DT_7 annealed to dA_{16} in TBE buffer following various manipulations. Lane 1, intact heteroduplex; lane 2, heteroduplex treated with NaOH to induce both β- and δ-eliminations; lane 3, reacted with endo III; lane 4, reacted with endo III and reduced with $NaBH_4$; lane 5, reacted with endo III and incubated with TBE; lane 6, reacted with endo III, incubated with TBE, and then reduced with $NaBH_4$; lane 7, reacted with endo III and incubated with 0.1 M Tris, pH 8.3; lane 8, reacted with endo III, incubated with 0.1 M Tris, pH 8.3, and then reduced with $NaBH_4$; lane 9, reacted with endo III and incubated with 0.1 M sodium borate, pH 8.3; lane 10, reacted with endo III, incubated with 0.1 M sodium borate, pH 8.3, and then reduced with $NaBH_4$. (A) The product of δ-elimination; (B and C) the β-elimination product; (D) the product of reduction of the β-elimination product in the presence of Tris; (E) intact starting material.

Characterization of an Electrophoresis Artifact in TBE Buffer. Fifteen micrograms of 5′-end labeled d(T_7DT_8) was annealed to 15 μg of d(A_{16}) at room temperature for 10 min in a total volume of 150 μL. The intact heteroduplex is electrophoresed in lanes 1 in Figures 1 and 2.

A 2-μg aliquot of labeled heteroduplex was treated with 100 mM NaOH for 1 h at 37 °C to effect a mixture of β- and δ-elimination reactions (lanes 2 in Figures 1 and 2). Ten 2-μg aliquots of the heteroduplex were separately treated with 500 ng of endo III for 1 h at 37 °C to effect complete strand cleavage. These aliquots were then further manipulated as described in the legends to Figures 1 and 2.

Scheme III



1, R = pdA
2, R = d(CGCAG)
3, R = pdG

4, R = pdA
5, R = d(CGCAG)
6, R = pdG

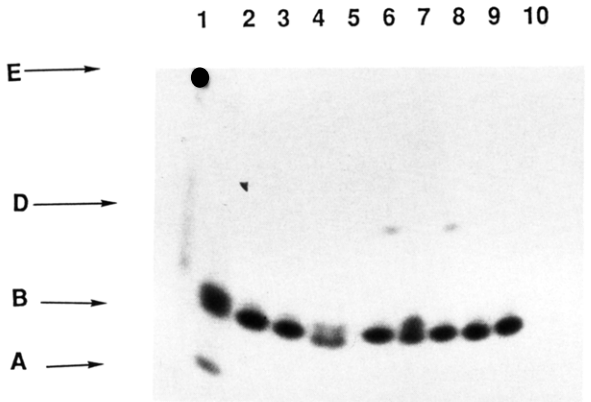


FIGURE 2: Electrophoresis of the reaction product obtained from [^{32}P]pdT$_8$DT$_7$ annealed to dA$_{16}$ in HEPES buffer following various manipulations. Lane 1, intact heteroduplex; lane 2, heteroduplex treated with NaOH to induce both β- and δ-eliminations; lane 3, reacted with endo III; lane 4, reacted with endo III and reduced with $NaBH_4$; lane 5, reacted with endo III and incubated with TBE; lane 6, reacted with endo III, incubated with TBE, and then reduced with $NaBH_4$; lane 7, reacted with endo III and incubated with 0.1 M Tris, pH 8.3; lane 8, reacted with endo III, incubated with 0.1 M Tris, pH 8.3, and then reduced with $NaBH_4$; lane 9, reacted with endo III and incubated with 0.1 M sodium borate, pH 8.3; lane 10, reacted with endo III, incubated with 0.1 M sodium borate, pH 8.3, and then reduced with $NaBH_4$. (A) The product of δ-elimination; (B) the β-elimination product; (D) the product of the reaction of the β-elimination product in the presence of Tris; (E) the intact starting material.

One-half of each aliquot was subjected to polyacrylamide gel electrophoresis in TBE buffer (0.1 M Tris, 0.1 M boric acid, and 2 mM EDTA, pH 8.3; Figure 1) and one-half in HE buffer (0.1 M HEPES–NaOH and 2 mM EDTA, pH 8.3; Figure 2). The results were analyzed by autoradiography.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 20% polyacrylamide gels [19:1 acrylamide–bis(acrylamide)] by using either TBE or HE buffer. The sample buffer was 95% deionized formamide containing 0.03% xylene cyanol, 0.03% bromphenol blue, and 20 mM EDTA, pH 7.0.

Determination of the Structures of β-Elimination Products by Reverse-Phase HPLC (Scheme III). Photoisomerization of α,β-unsaturated aldehydes esterified to 3′-ends of reaction products was accomplished by irradiation with flint-filtered light for 45 min. Following further degradation with nuclease P_1 but not alkaline phosphatase, the geometry of the double bond of a product (before and after photoisomerization) could be determined by comparing the HPLC retention time with standards of known geometry. The differing HPLC behavior for trans and cis isomeric products was first noted following degradation of the necessarily single-stranded poly(dA-D) with UV endo V (**1**, trans) and subsequent photoisomerization (**4**, cis). The structures of these materials have been determined by ^{1}H and ^{13}C NMR spectroscopies (Mazumder et al., 1989). UV endo V was subsequently used to degrade a polymeric double-stranded substrate with the same syn stereochemical course (Mazumder, 1990) reported for the single-stranded polymer (Mazumder et al., 1989). Therefore, we concluded that the stereochemical course of the reaction catalyzed by UV endo V was independent of the substrate and could be used to obtain the authentic trans (**2** prior to digestion and **3** after digestion with nuclease P_1) and cis (**5** prior to digestion and **6** after digestion with nuclease P_1) isomeric products from the synthetic 11-mer used as substrate in the studies described in this article; compounds **3** and **6** were used as chromatography standards.

After each degradation was complete (and the base-induced reaction was neutralized), the products were digested with nuclease P_1 followed by adenylic acid deaminase (1 unit) at 25 °C for 30 min to convert dAMP to dIMP (**3** coelutes with dAMP but not with dIMP). The resulting mixture of nucleotides and β-elimination product was separately chromatographed with **3** and **6**.

Stereospecificity of Hydrogen Abstraction. After uracil was removed from substrates that were stereospecifically labeled with ^{3}H in either the 2′-*pro-R* or 2′-*pro-S* hydrogen, sufficient endo III was added to achieve complete reaction in times ranging from 60 to 180 min. Aliquots were removed and quenched with $NaBH_4$ at several extents of reaction, and following degradation with nuclease P_1 and alkaline phosphatase, the amounts of unreacted abasic site and β-elimination product present and their specific radioactivities were measured. A similar procedure was used for the NaOH-induced degradation except that the pH was adjusted to 13 by the addition of an equal volume of 0.2 M NaOH.

In the case of the degradation induced by Lys-Trp-Lys, following complete reaction, reduction with $NaBH_4$, and digestion with both nuclease P_1 and alkaline phosphatase, the tritium content of both the solvent (volatile) and the reduced β-elimination product (nonvolatile) were quantitated following the bulb-to-bulb lyophilization. This procedure was used instead of the one described in the previous paragraph since Lys-Trp-Lys interfered with the HPLC separation.

Stereochemical Course of the Reaction Catalyzed by Ribonucleoside Triphosphate Reductase from L. leichmannii. The position of the hydrogen incorporated from solvent during the course of the enzymatic reduction of UTP to dUTP was reexamined according to the ^{1}H NMR procedure used by Batterham et al. (1967) except that the spectroscopy was performed at 400 MHz rather than 60 MHz. In addition, the nuclear Overhauser effect correlation spectrum of dUTP was obtained to ensure the validity of the chemical shift assignments of the 2′-*pro-R* and 2′-*pro-S* protons.

Results and Discussion

A Single Product Is Produced by β-Elimination Reactions of Abasic Sites. Conflicting results have been reported regarding the number of products obtained from both enzymatic and nonenzymatic β-elimination reactions. Verly and Bailly reported that oligonucleotides presumed to have an α,β-unsaturated aldose at their 3′-ends electrophorese as closely

spaced doublets in polyacrylamide gels run in TBE buffer (Bailly & Verly, 1987, 1988; Bailly et al., 1989a,b). In contrast, we observed a single α,β-unsaturated aldose product by ^{1}H NMR spectroscopy when the degradation of poly(dA-D) was catalyzed by UV endo V in phosphate buffer (Mazumder et al., 1989). Although Bailly and Verly have speculated that a pair of products may result from cis,trans-isomerism of the α,β-unsaturated aldose product, no evidence for this hypothesis was reported.

We hypothesized that the ability of aldehydes to form imines with Tris might offer an explanation for two products, the free aldehyde and the imine with Tris. Autoradiograms of various reactions of 5′-end labeled $d(T_7DT_8)$ annealed to $d(A_{16})$ and electrophoresed in TBE and HE buffers are displayed in Figures 1 and 2, respectively; these data support our hypothesis.

Duplex with the intact abasic site was electrophoresed in lane 1 (band E in Figures 1 and 2). Duplex treated with base or reacted with endo III in HEPES buffer was electrophoresed in lanes 2 or 3, respectively. When electrophoresed in TBE buffer, base treatment produces the doublet previously associated with the the β-elimination reaction (bands B and C in the Figure 1), as well as a faster migrating species with a 3′-phosphate end due to δ-elimination (band A); endo III produces only the β-elimination doublet. When electrophoresed in HE buffer (Figure 2), the same reactions produce only a single band for the β-elimination product (band B in Figure 2).

Given our hypothesis, the β-elimination product produced by endo III was reduced with $NaBH_4$ in the presence of HEPES buffer before electrophoresis. When electrophoresed in TBE or HE, only a single band is observed (lane 4), thereby demonstrating that in the absence of a primary amine, a single reaction product, the α,β-unsaturated aldehyde, is present (band B).

Additional evidence for the formation of an imine with Tris was obtained. The β-elimination product was mixed and incubated with TBE, 0.1 M Tris, pH 8.3, or 0.1 M sodium borate, pH 8.3, prior to reduction and electrophoresis. When the β-elimination product was incubated in the presence of Tris and electrophoresed without reduction (lanes 5 and 7), the characteristic doublet (bands B and C in Figure 1) is observed after electrophoresis in TBE, whereas a single band is observed after electrophoresis in HE (band B in Figure 2). If the reaction mixtures are reduced with $NaBH_4$ prior to electrophoresis (lanes 6 and 8), two bands of significantly different mobility are observed in both electrophoresis systems. The faster moving band (band B), attributed to the reduced aldehyde that retains the α,β-double bond (data not shown), has a mobility similar to the unreduced species, which we attribute to the aldehyde. The slower moving band (band D), presumably associated with the reduced imine, has a significantly lower mobility due to protonation at pH 8.3. Since the difference in pK_as of imines and amines is approximately 2–3 pH units, we attribute the higher mobility of the unreduced imine to its being unprotonated at pH 8.3. Incubation of the β-elimination product with sodium borate, the other major component of TBE, and electrophoresis without reduction (lane 9) yield the characteristic doublet in TBE (bands B and C in Figure 1) and a single band in HE (band B in Figure 2); incubation of the β-elimination product in sodium borate and reduction prior to electrophoresis yields a single product (lane 10) in both systems.

HPLC Method for Distinguishing the Geometric Isomers of the α,β-Unsaturated Aldehyde Product. The trans α,β-unsaturated aldose esterified to deoxyadenosine 3′,5′-bisphosphate (**1**, Scheme III) obtained by the action of UV endo V on the single-stranded alternating polymer of deoxyadenosine and abasic sites (Mazumder et al., 1989) and the cis isomer obtained by photoisomerization (**4**, Scheme III) can be resolved by HPLC (**1** elutes at 7.5 min and **4** at 6.5 min). In Figure 3, panel A, the chromatogram of **1** obtained from the reaction with UV endo V has been reproduced; in panel B, the chromatogram of **1** cochromatographed with **4** has been reproduced. No difference in retention times could be observed after reduction with $NaBH_4$ and/or removal of the 5′-phosphate group with alkaline phosphatase.

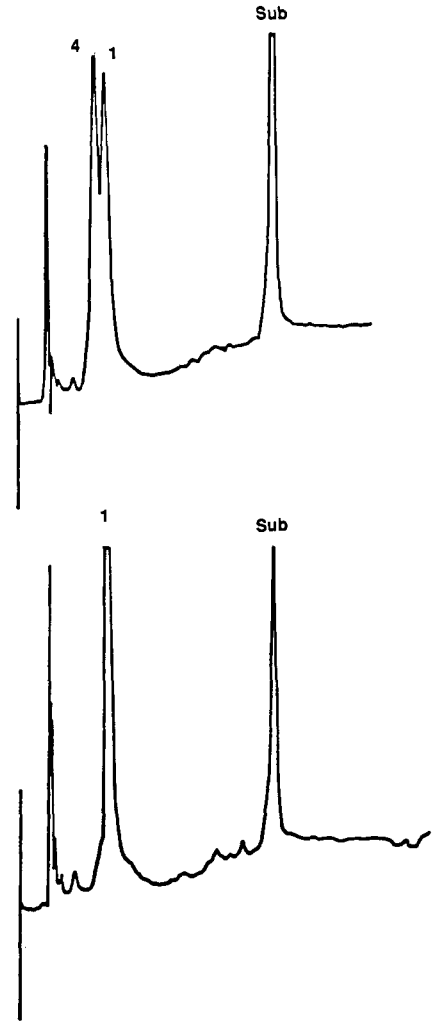


FIGURE 3: (A, Bottom) HPLC chromatogram of the product obtained from the partial degradation of poly(dA-D) by UV endo V. (B, Top) HPLC chromatogram obtained from cochromatography of the photoisomerized product with the trans product that was chromatographed in panel A. **1** and **4**, from Scheme III; Sub, the unreacted abasic site esterified to the 5′-phosphate group of deoxyadenosine 3′,5′-bisphosphate.

Since d(CGCAGDCAGCC) paired with d(GGCTGACTGCG), where D represents the aldehydic abasic site, was used in stereochemical studies reported in this article, it was necessary to distinguish the trans (**3**, Scheme III) and cis (**6**, Scheme III) geometric isomers of the enzymatic product. The trans product is obtained when UV endo V acts on a double-strand polymer containing abasic sites (Mazumder, 1990). Therefore, UV endo V was used to catalyze the degradation of the double-stranded undecameric oligonucleotide containing the abasic site. Following degradation with nuclease P_1, treatment with adenylate deaminase (since dAMP but not dIMP has the same retention time as **3**), and photoisomerization, the retention times of the trans (**3**) and

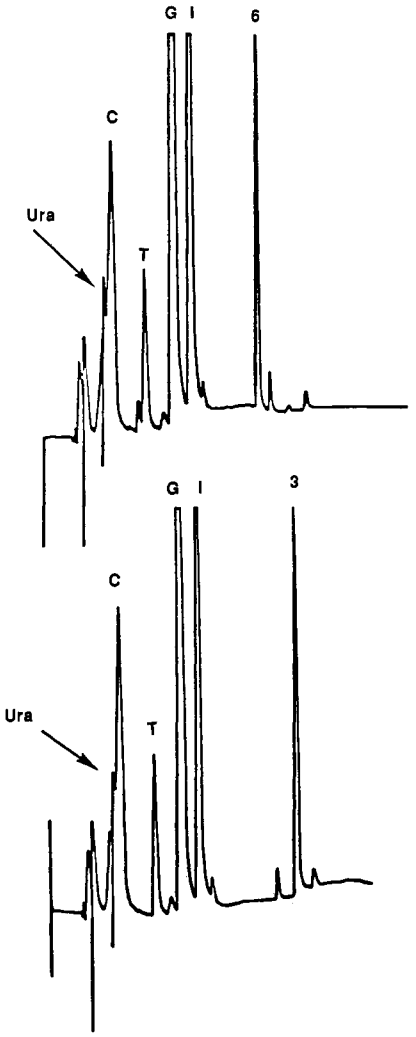


FIGURE 4: HPLC chromatograms of the product obtained from the degradation of the 11-mer by UV endo V, (A, bottom) before photoisomerization and (B, top) after photoisomerization. Ura, uracil; C, 5′-dCMP; T, 5′-TMP; G, 5′-dGMP; I, 5′-dIMP; **3** and **6**, from Scheme III.

cis (**6**) geometric isomers of the UV endo V product were measured; **3** elutes with a retention time of 21.5 min, and **6** elutes with a retention time of 19.3 min (Figure 4).

The β-Elimination Reaction Catalyzed by Endo III. After reaction of the undecameric substrate with endo III and further degradation to mononucleotide products with nuclease P_1, the structure of the α,β-unsaturated aldose product was determined by using the HPLC method described in the previous section (data not shown). The trans geometric isomer is produced by endo III.

Undecameric substrates were prepared with either the 2′-*pro-R* or the 2′-*pro-S* hydrogen of the abasic site stereospecifically labeled with ^3H and degraded with endo III. Following reduction with $NaBH_4$ and enzymatic degradation, the unreacted abasic site and β-elimination product were separated by HPLC. The specific radioactivities of each were quantitated as a function of the extent of reaction. As shown in Table I, the product obtained from the 2′-*pro-S* labeled substrate was not labeled; the specific radioactivity of the substrate was observed to be invariant with the extent of reaction. The product from the 2′-*pro-R* labeled abasic site had the same specific activity as the substrate.

The stereochemical course of the β-elimination reaction catalyzed by endo III is syn (Scheme I), since the geometry of the enzymatic product is trans and the 2′-*pro-S* hydrogen

Table I: Stereospecificity of 2′-Hydrogen Abstraction by Endo III

extent[a]	abasic site substr[b] (cpm/nmol)	β-elim prod[c] (cpm/nmol)	^3H_2O[d] (cpm)
	[2′-*pro-R*-^{3}H]-11-mer[e]		
0%	482		
40%	486	490	0
59%	481	481	0
100%		480	0
	[2′-*pro-S*-^{3}H]-11-mer[f]		
0%	2520		
64%	2480	0	9416
86%	2500	0	12408
100%		0	14500

[a] Reaction extent measured by percent conversion of substrate to product. [b] Specific radioactivity of the recovered substrate. [c] Specific radioactivity of the recovered product. [d] Radioactivity recovered in the column flow through in cpm. [e] The reaction contained 9.5 nmol of 11-mer. [f] The reaction contained 5.6 nmol of 11-mer.

is abstracted. This is identical with the stereochemical course of the reaction catalyzed by UV endo V on both single-stranded and double-stranded substrates (Mazumder et al., 1989; Mazumder, 1990). No ^3H selection effect is observed in the reaction catalyzed by endo III or in the reactions catalyzed by UV endo V on double-stranded but not single-stranded substrates (Mazumder et al., 1989; Mazumder, 1990). The selection of 8 we reported earlier for the degradation of a single-stranded polymeric substrate by UV endo V was miscalculated (Mazumder et al., 1989); the correct value is 1.5.

These syn stereochemical courses are the same as those documented for a number of other enzymes catalyzing β-eliminations from carbonyl compounds and thiolesters (Schwab et al., 1986; Widlanski et al., 1987). Since this stereochemical course requires that the anionic phosphodiester leaving group be on the same side of the $C_{2'}$–$C_{3'}$ bond as the proton that is abstracted to initiate the reaction, it is possible that the phosphate ester may be catalyzing its own elimination. Such a mechanism has been hypothesized in at least two examples of enzyme-catalyzed eliminations of inorganic phosphate (Gallopo & Cleland, 1979; Widlanski et al., 1989). The syn stereochemical course also demonstrates that the elimination reaction cannot proceed from the cyclic hemiacetal tautomeric form of the aldehydic abasic site but must proceed from either the aldehyde tautomer or an activated imine derived from the aldehyde. If the internucleotidic phosphodiester is catalyzing its own β-elimination, it is probable that the reactive species is the protonated imine to allow the 2′-*pro-S* proton to have a sufficiently low pK_a such that it can be abstracted by the poorly basic phosphodiester anion.

The stereospecificities of hydrogen abstraction we have observed in the reactions catalyzed by both UV endo V and endo III differ from those implicitly determined in Bailly and Verly's studies (Bailly & Verly, 1987; Bailly et al., 1989a). Since the position of the ^3H in our substrates depends upon the stereochemical course of the reaction catalyzed by ribonucleoside triphosphate reductase, we redetermined it in the course of our studies and found that it had retained its configuration as had been originally reported (data not shown).

The finding that endo III contains a 4Fe–4S center could suggest that this cluster may be involved in the reactions catalyzed by the enzyme. UV endo V has no chromophore that absorbs in the visible region of the spectrum (data not shown); thus, it does not contain a similar 4Fe–4S center (Switzer, 1989). The identical stereochemical consequences of the β-elimination reactions catalyzed by UV endo V and endo III demonstrate that the cluster need not have a mech-

of unreacted cyclic structures. Thus, the observed trans product in these nonenzymatic reactions is expected. The observations that the reaction induced by NaOH is not accompanied by a decrease in the specific radioactivity of the substrate, that no ^3H is found in the product obtained from the 2′-*pro-R* labeled substrate, that the specific radioactivity of the product obtained from the 2′-*pro-S* labeled substrate remains constant and equal to the specific radioactivity of the initial substrate, and that the specific radioactivity of unreacted 2′-*pro-R* labeled substrate increases as the reaction progresses all indicate that once the proton is abstracted from the 2′-position the reaction is committed to elimination of the 3′-phosphodiester. Thus, this reaction is likely to occur via either an E_2 or an irreversible E_1cb mechanism.

Conclusions. The reactions catalyzed by UV endo V and endo III proceed by the same syn stereochemical course even though endo III contains a 4Fe–4S center. Thus, enzyme-catalyzed β-elimination reactions of aldehydic abasic sites are not unique to Fe–S-containing enzymes. Furthermore, the observation that the chemical and enzymatic reactions proceed with different stereochemical consequences suggests that the syn stereochemical courses established for the enzymatic reactions may reflect a mechanistic imperative in which the basicity of the phosphodiester leaving group and the acidity of the 2′-*pro-S* hydrogen are exploited to allow the phosphodiester to catalyze its own β-elimination (Gallopo & Cleland, 1979; Widlanski et al., 1989).

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