# Reaction Mechanism of T4 Endonuclease V Determined by Analysis Using Modified Oligonucleotide Duplexes†

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ABSTRACT: The reaction mechanism of bacteriophage T4 endonuclease V was investigated using modified oligodeoxyribonucleotide duplexes containing a cis-syn thymine dimer. For the pyrimidine dimer glycosylase step, the formation of a covalent intermediate has been proposed. A fluorine atom was attached to the 2′-position of the 5′-component of the thymine dimer site, which could stabilize the covalent complex and prevent the ring opening of the sugar moiety. The strand cleavage of the 12 base pair substrate analog did not occur, although the glycosyl bond was cleaved by this enzyme. A covalent enzyme−substrate complex was separated by gel electrophoresis under denaturing conditions. It was shown that the enzyme molecules were completely converted to a stable complex in the reaction mixture. Two mechanisms have been proposed for the β-elimination step. A 12-mer containing a phosphorothioate linkage between adjacent thymidines was prepared. The diastereomers were separated, and the absolute configurations were determined. After formation of the thymine dimer and $^{32}$P-labeling of the 5′-terminus, these oligonucleotides were annealed to the complementary 12-mer, and the reaction rates of the pyrimidine dimer glycosylase step and the overall reaction for each duplex were measured under the substrate-saturation conditions. The rate constants indicated that the chemical reaction at the β-elimination step was rate-limiting. Since no difference was observed in the rate constants for the $R_P$- and $S_P$-phosphorothioate substrates, it is concluded that the β-elimination reaction is catalyzed, not by the internucleotide phosphate, but by an amino acid residue of the enzyme.

Endonuclease V from bacteriophage T4 initiates the repair of DNA lesions caused by ultraviolet (UV)[1] light. After the nonspecific binding to DNA, the enzyme scans along the DNA duplex processively to search for the target (Ganesan et al., 1986; Gruskin & Lloyd, 1986, 1988) and binds specifically to the cis-syn cyclobutane pyrimidine dimer which is caused by the UV irradiation. In our previous work, we demonstrated that this specific binding occurred in the minor groove of the substrate duplex (Iwai et al., 1994). The first reaction catalyzed by this enzyme is the hydrolysis of the glycosyl bond of the 5′-component of the dimer. It has been proposed that a covalent intermediate is formed between the N-terminal α-amino group of the enzyme and the C1′ of the 5′-component of the pyrimidine dimer at this glycosylase step (Figure 1) (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993). It was shown that mutation of the glutamic acid at position 23 resulted in a loss of the glycosylase activity, although this mutant retained the property of the specific binding (Doi et al., 1992). The second step is the strand cleavage at the resultant apyrimidinic site by a β-elimination mechanism (Manoharan et al., 1988, 1989). It has been reported that this reaction proceeds by a syn β-elimination involving the abstraction of the 2′-*pro*-*S*-hydrogen and the formation of a trans α,β-unsaturated product (Mazumder et al., 1989, 1991). Two mechanisms have been proposed for this catalytic reaction (Figure 1). In one of them, the internucleotide phosphate residue 3′ to the abasic site was assumed to abstract the 2′-hydrogen (Schrock & Lloyd, 1991; Mazumder et al., 1991). In contrast, our previous data suggested that the glutamate at position 23 of the enzyme might be the general base in this reaction (Hori et al., 1992).

T4 endonuclease V is a well-characterized repair enzyme. This enzyme was crystallized, and its tertiary structure was determined by X-ray diffraction (Morikawa et al., 1992). The structure−function relationship of the enzyme has been studied intensively by site-directed mutagenesis (Doi et al., 1992; Recinos & Lloyd, 1988; Stump & Lloyd, 1988; Dowd & Lloyd, 1989a,b, 1990; Ishida et al., 1990; Green et al., 1993; Latham et al., 1994). The structure of DNA containing a pyrimidine dimer, which is the substrate for this enzyme, has been investigated mainly by nuclear magnetic resonance (NMR) spectroscopy (Kemmink et al., 1987a,b; Taylor et al., 1990; Lee et al., 1994). However, the enzyme−substrate interactions and reaction mechanisms have not been elucidated completely. For elucidation of these interactions and mechanisms, it is necessary to utilize substrate analogs which are modified at the pyrimidine dimer site.

In this article, we describe an analysis of the reactions catalyzed by T4 endonuclease V using synthetic oligonucleotide duplexes. Two kinds of duplexes were prepared to analyze the two-step enzyme reaction. The first one contained a fluorine atom at the 2′-position of the 5′-component of the thymine dimer site (Figure 2a). This

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[1] Abbreviations: UV, ultraviolet; NMR, nuclear magnetic resonance; $T_F$, 2′-fluorothymidine; DMT, 4,4′-dimethoxytrityl; T[]T, *cis-syn* thymine dimer; PS, the phosphorothioate linkage; TEAA, triethylammonium acetate; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PD, pyrimidine dimer; T[PS]T, *cis-syn* thymine dimer with a phosphorothioate linkage at this site.

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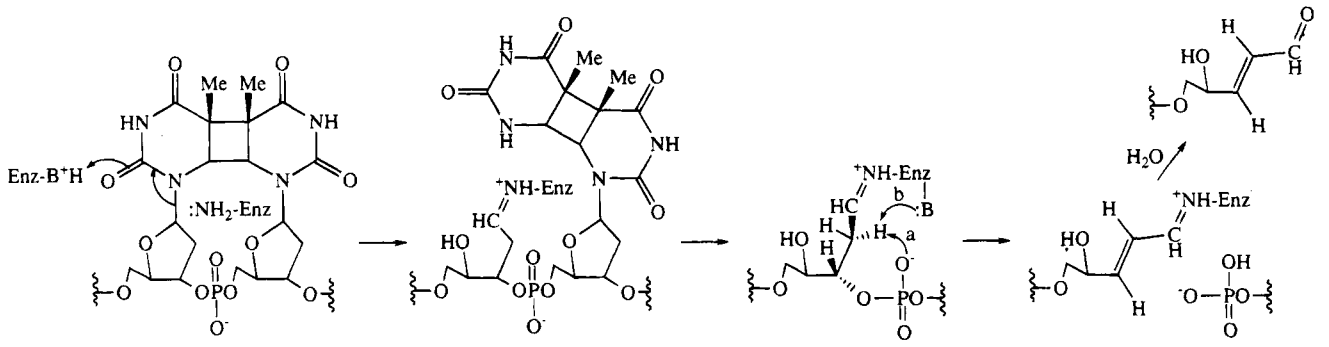

FIGURE 1: Proposed mechanism for the endonuclease V reaction. Either the internucleotide phosphate of the substrate (a) or an amino acid residue of the enzyme (b) abstracts the 2′-hydrogen in the β-elimination.

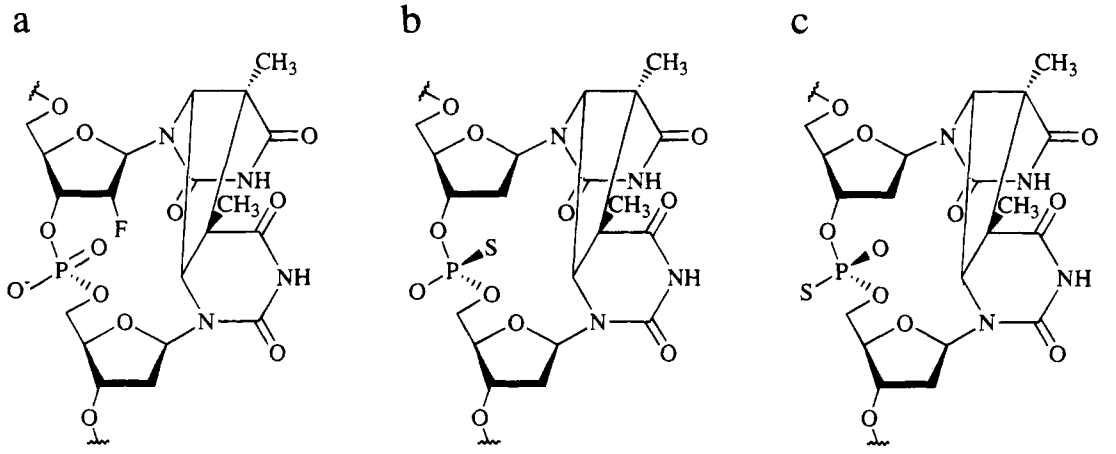

FIGURE 2: Structures of the modified residues used in this study, 2′-fluorothymidine (a) and the phosphorothioate linkages with the $R_P$ (b) and $S_P$ (c) configurations at the thymine dimer site. These were incorporated into the d(GCACGT[]TGCACG) sequence.

substrate analog was designed to detect the proposed covalent intermediate as an intact form, in expectation of the stabilization of the covalent bond by the electron-withdrawing fluorine atom on the neighboring carbon. The enzyme reaction with this analog was analyzed, and a covalent complex was separated by denaturing gel electrophoresis. The second duplex contained a phosphorothioate linkage at the thymine dimer site, in which one of the nonbridging oxygens of the phosphodiester was replaced by a sulfur atom (Figure 2b,c), and was used to identify the general base in the β-elimination reaction. The diastereomers were annealed to the complementary strand separately, and the reaction rates of the isomers were compared under the saturation conditions, which compensate for differences in the substrate affinity for the enzyme.

## EXPERIMENTAL PROCEDURES

*Preparation of Oligonucleotides.* According to the method described by Williams et al. (1991), 2′-fluorothymidine ($T_F$) was synthesized, and its 5′- and 3′-hydroxyl functions were protected with the 4,4′-dimethoxytrityl (DMT) group and phosphitylated with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, respectively. Using this compound, a dinucleotide coupling unit containing a thymine dimer (the protected $T_F$[]T phosphoramidite) was prepared as described previously (Taylor et al., 1987; Murata et al., 1990). The chain assembly was carried out on an Applied Biosystems 394 DNA/RNA synthesizer with the 1.0-μmol synthesis cycle supplied by the manufacturer. The reagents for the synthesizer were purchased from Applied Biosystems. Four 12-mers, d(GCACGT[]TGCACG), d(GCACGT$_F$[]TGCACG), d(GCACGT$_{PS}$TGCACG), and d(CGTGCAACGTGC), where T[]T and $_{PS}$ represent the cis-syn thymine dimer and the phosphorothioate linkage, respectively, were synthesized for this study. The 12-mers containing the cis-syn thymine dimer with a phosphodiester linkage, d(GCACGT[]TGCACG) and d(GCACGT$_F$[]TGCACG), were prepared using a thymine dimer coupling unit (Murata et al., 1990). The phosphorothioate linkage was formed by sulfurization of the phosphite triester with tetraethylthiuram disulfide in acetonitrile (Vu & Hirschbein, 1991). This sulfurization was performed prior to the capping of the 5′-hydroxyl function with acetic anhydride. The DMT group at the 5′-end was not removed on the synthesizer. After the chain assembly and the cleavage from the support with aqueous ammonia, the ammoniacal solutions were heated at 55 °C for 5 h. Ammonia was removed by evaporation, and the dimethoxytritylated oligonucleotides were purified by chromatography on alkylated silica gel (preparative C18 125Å, Millipore Corporation). Elution was performed with a linear gradient of acetonitrile (from 0 to 40%) in 0.1 M triethylammonium acetate (TEAA, pH 7.0). After evaporation and coevaporation with water, the DMT group was removed with 80% aqueous acetic acid. The fully deprotected oligonucleotides were purified by reversed-phase high-performance liquid chromatography (HPLC), using an Inertsil ODS-2 column (10 × 250 mm, GL Sciences Inc.) with a linear gradient of acetonitrile in 0.1 M TEAA. At this step, the two diastereomers of d(GCACGT$_{PS}$TGCACG) were separated, with a gradient of acetonitrile (from 9 to 10% during 20 min) (Figure 3a). The purity of each oligonucleotide was analyzed by anion-exchange HPLC, using a TSK-GEL DEAE-2SW

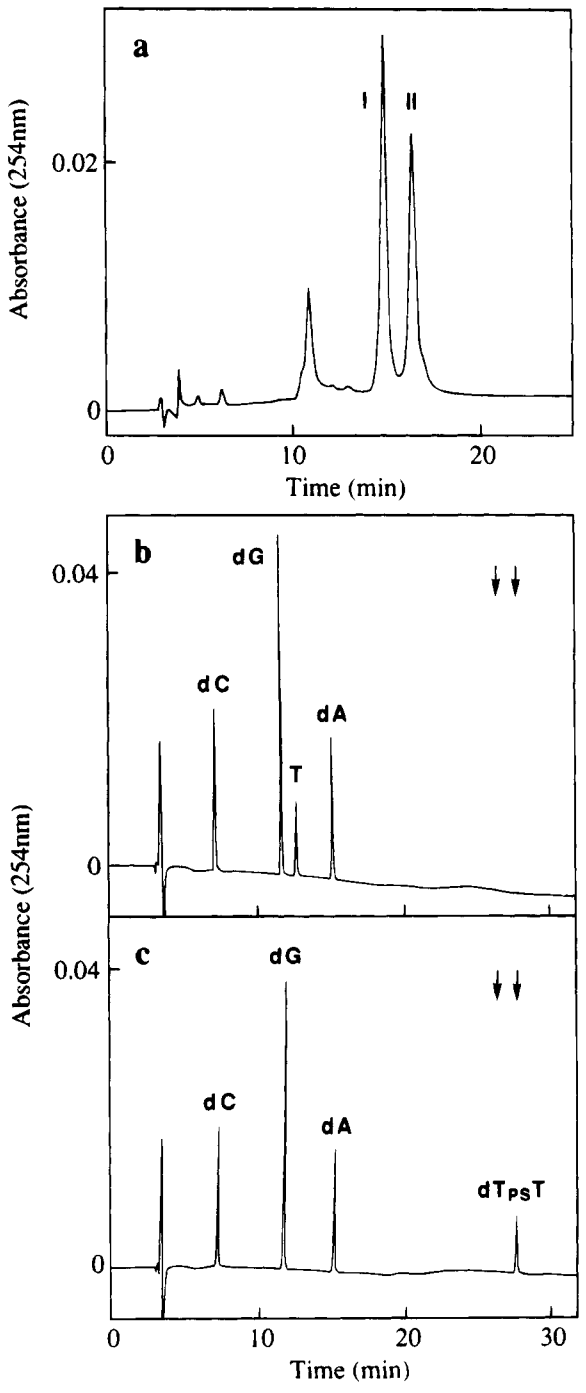


FIGURE 3: (a) Elution profiles of the crude d(GCACGT$_{PS}$TGCACG). Peaks I and II are the diastereomers. (b and c) Analysis of the digested products of peaks I (b) and II (c). The arrows indicate the retention times of the two isomers of d(T$_{PS}$T).

column (4.6 × 250 mm, Tosoh Corporation) with a linear gradient of ammonium formate in 20% aqueous acetonitrile. The molar concentration of each product was calculated as described (Borer, 1975).

*Enzymatic Digestion of the Oligonucleotides.* The separated diastereomers (1.0 $A_{260}$ unit) were dissolved in 39 μL of water and 10 μL of 0.25 M Tris-HCl (pH 8.0) containing 50 mM $MgCl_2$, and snake venom phosphodiesterase (1 μL, 2 μg, Boehringer Mannheim) was added. The mixtures were incubated at 37 °C for 14 h, and alkaline phosphatase (from calf intestine, 1 μL, 1 unit, Boehringer Mannheim) was added. After incubation at 37 °C for 1 h and dilution to 250 μL, aliquots (5 μL) of the mixtures were analyzed by reversed-phase HPLC, using a YMC AP-303 column (4.6 × 250 mm, Yamamura Chemical Laboratories) at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile (from 0 to 15% during 30 min) in 0.1 M TEAA (Figure 3b,c). Dithymidine phosphorothioate, d(T$_{PS}$T), prepared on the synthesizer and deprotected with trichloroacetic acid and then with aqueous ammonia, was eluted under the same conditions.

*Formation of the Thymine Dimer.* The thymine dimer was formed in d(GCACGT$_{PS}$TGCACG) by UV irradiation using a 100-W mercury lamp, as described previously (Inaoka et al., 1989). After irradiation of the solutions (1.0 $A_{260}$) through filters of colored glass (Toshiba UV-25) and a $NiSO_4$ solution for 2.5 h, aliquots were analyzed by reversed-phase HPLC, using an Inertsil ODS-2 column (4.6 × 250 mm) with a linear gradient of acetonitrile (from 8 to 11% during 20 min) in 0.1 M TEAA, and the photoproducts were isolated under the same conditions. Each product was assayed for the thymine dimer by reirradiation and detection of the reversed product by HPLC.

*Preparation of Labeled Duplexes.* The oligonucleotides containing a thymine dimer (20 pmol) were labeled with [γ-$^{32}$P]ATP (1 × $10^7$ cpm, New England Nuclear) and T4 polynucleotide kinase (10 units, Takara Shuzo) in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 5 mM dithiothreitol (10 μL). The mixtures were incubated at 37 °C for 1 h, diluted with water to 200 μL, and passed through a NENSORB 20 column (DuPont). After concentration, the labeled oligonucleotides were mixed with the respective unlabeled thymine dimer oligonucleotide to adjust the radioactivity, and a 1.2-fold excess of the complementary 12-mer, d(CGTGCAACGTGC), was added. These mixtures were heated at 75 °C and cooled to room temperature.

*Glycosyl Bond and Strand Cleavage by T4 Endonuclease V.* The oligonucleotide duplexes (2 pmol, 1 × $10^4$ cpm) were dissolved in a reaction buffer (10 μL) containing 32 mM Tris-HCl (pH 7.5), 9.6 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 M NaCl, and were mixed with a 2 μM solution (10 μL) of T4 endonuclease V, produced and purified as described previously (Inaoka et al., 1989), in 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA, 0.1 M NaCl, and 0.02% bovine serum albumin. These mixtures were incubated at 30 °C for 30 min. For the complete strand cleavage of the abasic intermediates, 0.15 M aqueous piperidine (40 μL) was added, and the mixtures were heated at 95 °C for 30 min. Piperidine was removed by evaporation and coevaporation with water. The products were separated by 20% polyacrylamide gel electrophoresis (PAGE) containing 7 M urea.

*Separation of the Covalent Intermediate.* The above reaction mixture (20 μL) containing [$^{32}$P]d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC) was incubated at 30 °C for 30 min, and, without the piperidine treatment, a solution (20 μL) containing 10% sodium dodecyl sulfate (SDS), 20% glycerol, 20% mercaptoethanol, 0.02% bromophenol blue, and 125 mM Tris-HCl (pH 6.8) was added. For reference, [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) was treated with the enzyme under the $NaBH_4$-reducing conditions as described by Dodson et al. (1993). These mixtures were subjected to separation by 15% SDS−PAGE.

*Inactivation of the Enzyme by the Fluorine-Containing Substrate Analog.* The unlabeled duplexes (20 pmol), d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) and d(G-

CACGT$_F$[]TGCACG)•d(CGTGCAACGTGC), were dissolved in the reaction buffer (10 $\mu$L) and mixed with a 2 $\mu$M solution (10 $\mu$L) of T4 endonuclease V. After incubation at 30 °C for 20 min, [$^{32}$P]d(GCACGT[]TGCACG)•d(CGTGCAACGTGC) (2 pmol) was added to each mixture. These mixtures were incubated for another 20 min, and the products were separated by 20% PAGE.

*Native Gel Electrophoresis.* The reaction mixture contained [$^{32}$P]d(GCACGT$_F$[]TGCACG)•d(CGTGCAACGTGC) (20 pmol) and T4 endonuclease V (20 pmol) in the reaction buffer (20 $\mu$L). After incubation at 30 °C for 30 min, 40% sucrose containing 0.25% bromophenol blue was added, and the mixture was loaded onto a 6% polyacrylamide gel containing 6.7 mM Tris-HCl (pH 8.0) and 3.3 mM sodium acetate. Electrophoresis was performed in a 4 °C room at 4 V/cm for 4 h with the circulation of the anode and cathode buffers (6.7 mM Tris-HCl (pH 8.0) and 3.3 mM sodium acetate).

*Kinetic Analysis of the T4 Endonuclease V Reaction.* The oligonucleotide duplexes ($2 \times 10^3$ cpm) were dissolved in the reaction buffer (15 $\mu$L) and were mixed with a solution (15 $\mu$L) of T4 endonuclease V. For [$^{32}$P]d(GCACGT[]TGCACG)•d(CGTGCAACGTGC) and the $R_P$-isomer of [$^{32}$P]d(GCACGT[$_{PS}$]TGCACG)•d(CGTGCAACGTGC), the substrate concentration was varied from 20 to 50 nM, at the enzyme concentration of 15 nM. For the $S_P$-isomer, the substrate and enzyme concentrations were 50−200 and 67 nM, respectively. These mixtures were incubated at 30 °C for 12 min, and 0.15 M aqueous piperidine (60 $\mu$L) was added. After being heated at 95 °C for 30 min, piperidine was removed by evaporation and coevaporation with water, and the products were separated by 20% PAGE. The gel was dried, and the radioactivity of each band was quantified on a FUJIX BAS2000 Bio-imaging Analyzer (Fuji Photo Film).

*Reaction Rates under the Substrate-Saturation Conditions.* Each oligonucleotide duplex ($1 \times 10^5$ cpm) was mixed with a $^{32}$P-labeled 24-mer ($2 \times 10^3$ cpm), [$^{32}$P]d(TCCTTCGTGATACCCGCGTCACAG), and was dissolved in the reaction buffer (10 $\mu$L). The concentrations of [$^{32}$P]d(GCACGT[]TGCACG)•d(CGTGCAACGTGC) and the $R_P$- and $S_P$-isomers of [$^{32}$P]d(GCACGT[$_{PS}$]TGCACG)•d(CGTGCAACGTGC) were 10, 5, and 72 $\mu$M, respectively. A 12 nM solution of T4 endonuclease V (10 $\mu$L) was added, and the mixtures were incubated at 30 °C. After 10, 20, 30, and 40 min, aliquots (4 $\mu$L) were taken. For the overall reaction, they were mixed with 90% formamide (2 $\mu$L) containing the Tris−borate−EDTA buffer, 0.02% xylene cyanol, and 0.02% bromophenol blue. For the pyrimidine dimer (PD) glycosylase reaction, 0.15 M aqueous piperidine (20 $\mu$L) was added, and after being heated at 95 °C for 30 min and coevaporation with water, the residue was dissolved in a mixture of water (4 $\mu$L) and the formamide solution (2 $\mu$L). The products were separated by 20% PAGE, and the radioactivity was quantified on the imaging analyzer. Sampling errors were corrected using the 24-mer internal standard.

## RESULTS

*Endonuclease V Reaction with the 2′-Fluorinated Substrate Analog.* An oligodeoxynucleotide containing a fluorine atom at the 2′-position of the 5′-component of the thymine dimer site, d(GCACGT$_F$[]TGCACG) (Figure 2a), was prepared using a dinucleotide coupling unit in a 6.5% yield from deoxyguanosine on a solid support. The incorporation of 2′-fluorothymidine into the sequence was confirmed by digestion of the UV-irradiated oligonucleotide with snake venom phosphodiesterase and alkaline phosphatase, followed by HPLC analysis, as described (Fowler et al., 1982). After $^{32}$P-labeling, this thymine dimer 12-mer was annealed to the complementary strand, d(CGTGCAACGTGC), and the glycosyl bond cleavage and the strand scission by T4 endonuclease V were analyzed with and without the hot piperidine treatment, respectively. The products were separated by denaturing 20% PAGE, and the results are shown in Figure 4. The 12 base pair duplex without the sugar modification, [$^{32}$P]d(GCACGT[]TGCACG)•d(CGTGCAACGTGC), was cleaved completely to produce the $\beta$-elimination product (lane 3), and the $\delta$-elimination product was obtained upon the piperidine treatment (lane 4). In the case of the [$^{32}$P]d(GCACGT$_F$[]TGCACG)•d(CGTGCAACGTGC) duplex, no strand cleavage was observed without the piperidine treatment. Instead, an extra band was detected at the top of the gel (lane 7). The $\beta$- and $\delta$-elimination products were obtained when the sample was treated with piperidine (lane 8).

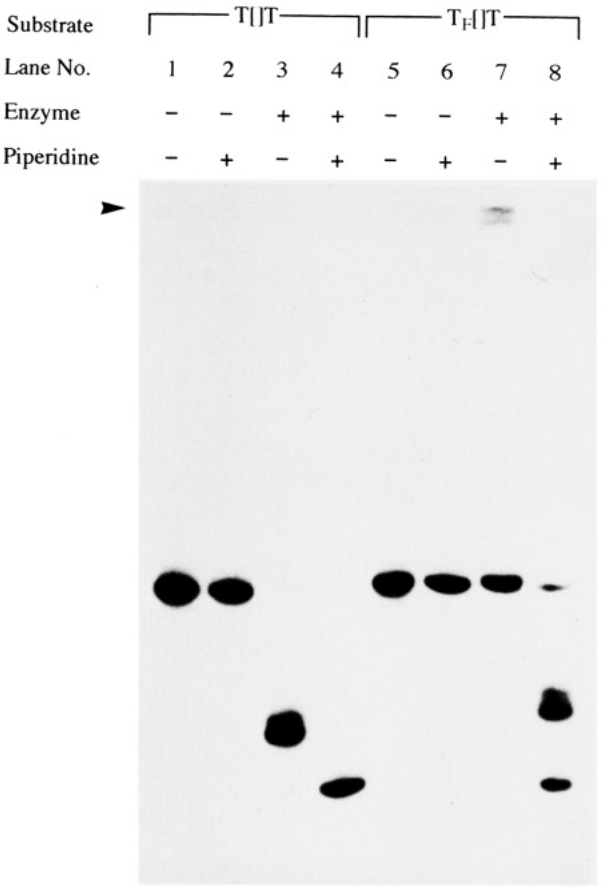


FIGURE 4: Analysis of the endonuclease V reaction with [$^{32}$P]d(GCACGT[]TGCACG)• d(CGTGCAACGTGC) (lanes 1−4) and [$^{32}$P]d(GCACGT$_F$[]TGCACG)•d(CGTGCAACGTGC) (lanes 5−8) by denaturing 20% PAGE. Lanes 1 and 5, no treatment; lanes 2 and 6, treated with piperidine; lanes 3 and 7, incubated with T4 endonuclease V; lanes 4 and 8, incubated with the enzyme and then treated with piperidine. The arrowhead indicates the top of the gel.

*Detection of the Covalent Complex by SDS−PAGE.* If the 2′-fluorine atom can stabilize the covalent bond between

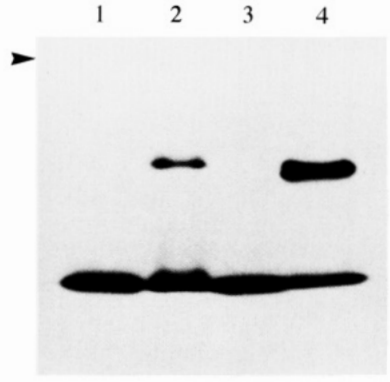


FIGURE 5: Separation of the covalent complex by SDS−PAGE. Lanes 1 and 3, the 12 base pair duplexes; lane 2, a mixture of T4 endonuclease V and [$^{32}$P]d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC); lane 4, a mixture of the enzyme and [$^{32}$P]d(GCACGT[]TGCACG)· d(CGTGCAACGTGC) in the presence of 0.2 M $NaBH_4$. The arrowhead indicates the top of the separating gel.

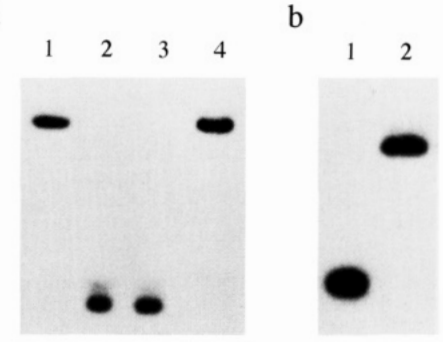


FIGURE 6: (a) The labeled substrate, [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC), was treated with T4 endonuclease V preincubated with an equimolar amount of unlabeled d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) (lane 3) or d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC) (lane 4). Lanes 1 and 2 show the intact substrate and the cleaved product, respectively. (b) Mobility shift of [$^{32}$P]d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC) on a nondenaturing gel. Lane 1, the duplex alone; lane 2, a 1:1 mixture of the enzyme and the duplex.

the neighboring C1′ carbon and the enzyme, the extra band with almost no mobility obtained in the reaction mixture containing [$^{32}$P]d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC) (Figure 4, lane 7) may well be the covalent complex. In order to make the mobility of this band greater, an SDS−PAGE experiment was carried out. The reaction mixture used for lane 7 in Figure 4 was prepared again and applied to a 15% polyacrylamide gel containing SDS. For reference, [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) was treated with T4 endonuclease V under the $NaBH_4$-reducing conditions, as described by Dodson et al. (1993), and this sample was loaded onto the same gel. The results are shown in Figure 5. In both cases, a slowly migrating band was detected by autoradiography, although the shift was not complete. In the case of the fluorine-containing analog, smearing was observed between the two bands when the X-ray film was exposed longer. The proportion of the shifted band to the total radioactive substance did not change as the enzyme concentration was increased (data not shown).

*Complex Formation in the Reaction Mixture.* The incomplete band shift in the SDS−PAGE experiment could be due to hydrolysis of the complex during electrophoresis. To determine whether the stable covalent complex of the enzyme with the fluorine-containing duplex was formed quantitatively in the solution, the strand cleavage of [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) by endonuclease V preincubated with an equimolar amount of unlabeled d(GCACGT$_F$[]TGCACG)·d(CGTGCAACGTGC) was analyzed. It was found that the labeled substrate was not incised in this reaction mixture, while the enzyme pretreated with the unmodified substrate was fully active (Figure 6a). The complete complex formation in the 1:1 mixture of the $^{32}$P-labeled fluorine-containing duplex and the enzyme was confirmed by non-denaturing 6% PAGE, as shown in Figure 6b.

*Oligonucleotides Containing a Phosphorothioate Linkage.* A phosphorothioate diester (Figure 2b,c) was used as the internucleotide linkage between the two adjacent thymidines in the sequence of d(GCACGTTGCACG), in order to study the mechanism of the $\beta$-elimination in the endonuclease V reaction. This linkage was formed by sulfurization of the phosphite triester with tetraethylthiuram disulfide, after coupling of the second thymidine phosphoramidite on a solid support (Vu & Hirschbein, 1991). After the chain assembly on a synthesizer and deprotection with ammonia, the oligonucleotide with a DMT group at the 5′-end was partially purified by chromatography on alkylated silica gel, and the DMT group was removed with acetic acid. As shown in Figure 3a, two products (peaks I and II) were separated by reversed-phase HPLC. Since these products could not be separated by anion-exchange HPLC and had the same mobility by PAGE, they were regarded as the diastereomers of d(GCACGT$_{PS}$TGCACG).

These isomers were degraded with snake venom phosphodiesterase and alkaline phosphatase to their nucleoside components. In the case of isomer I (peak I in Figure 3a), four peaks, corresponding to deoxycytidine, deoxyguanosine, thymidine, and deoxyadenosine, were obtained in a molar ratio close to 2:2:1:1, respectively (Figure 3b). On the other hand, thymidine was not detected in the products from isomer II (Figure 3c). Instead, an extra peak appeared, and its retention time was the same as the longer retention time of the dithymidine phosphorothioate diastereomer. It has been noted that the $S_P$-isomer of the phosphorothioate linkage is cleaved much more slowly than the $R_P$-isomer by snake venom phosphodiesterase (Burgers & Eckstein, 1979), and it also has been reported that the retention time of the $S_P$-isomer of dithymidine phosphorothioate is longer than that of the $R_P$-isomer by reversed-phase HPLC (Stec et al., 1985). Therefore, the absolute configurations of the phosphorothioate linkages in isomers I and II (peaks I and II in Figure 3a) were assigned to $R_P$ and $S_P$, respectively.

*Determination of the Kinetic Parameters.* The thymine dimer was formed in $R_P$- and $S_P$-d(GCACGT$_{PS}$TGCACG) by UV irradiation, and the products were purified by reversed-phase HPLC, as described previously (Inaoka et al., 1989). The formation of the cyclobutane dimer was confirmed by the photoreversion assay, because only the cyclobutane-type photoproducts could be reversed to the starting material, d(GCACGT$_{PS}$TGCACG), which had a longer retention time than d(GCACGT[$_{PS}$]TGCACG). The cis-syn structure was determined later by the endonuclease V cleavage. The $R_P$- and $S_P$-isomers of d(GCACGT[$_{PS}$]TGCACG) were labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase and annealed to the complementary strand, d(CGTGCAACGTGC).

Table 1: Kinetic Parameters of the PD Glycosylase Reaction

| substrate | $K_m$ (M) | $k_{cat}$ ($min^{-1}$) |
|---|---|---|
| d(GCACGT[]TGCACG)<br>d(CGTGCA ACGTGC) | $6.3 \times 10^{-8}$ | 1.84 |
| $R_P$-d(GCACGT[PS]TGCACG)<br>d(CGTGCA ACGTGC) | $2.8 \times 10^{-8}$ | 0.54 |
| $S_P$-d(GCACGT[PS]TGCACG)<br>d(CGTGCA ACGTGC) | $3.7 \times 10^{-7}$ | 0.88 |

Using these duplexes and the parent duplex containing a phosphodiester linkage at the thymine dimer site, [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC), as substrates for T4 endonuclease V, the kinetic parameters of the PD glycosylase reaction were determined. Each substrate duplex was incubated with the enzyme under conditions where the ratio of the cleaved product was less than 25%, and then the reaction mixtures were treated with hot piperidine to complete the strand cleavage at the abasic site. The products were separated by denaturing 20% PAGE, and the radioactivity of each band was quantified. Velocities ($v$) at varying substrate concentrations ([S]) were calculated, and the kinetic parameters were obtained from the double-reciprocal ($1/v$ versus $1/[S]$) Lineweaver–Burk plots (Table 1).

In order to confirm the difference in the affinity for the enzyme, the dissociation constants (the $K_d$ values) for the complexes were obtained by the filter-binding method (Iwai et al., 1994). The values for d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) and the $R_P$- and $S_P$-isomers of d(GCACGT[PS]TGCACG)·d(CGTGCAACGTGC) were $1.1 \times 10^{-8}$, $5.3 \times 10^{-9}$, and $1.4 \times 10^{-7}$ M, respectively.

*Rate Constants under the Substrate-Saturation Conditions.* In order to cancel the difference in the affinities of the $R_P$- and $S_P$-isomers of d(GCACGT[PS]TGCACG)·d(CGTGCAACGTGC) for the enzyme, the rate constants were determined under the conditions where the concentrations of the substrates were extremely high. Actually, substrate concentrations about 100-fold higher than each $K_m$ value were used. Since the amounts of the products must be measured accurately in these experiments, a $^{32}$P-labeled 24-mer, with a sequence that was not related to the substrate, was mixed with the substrate duplex prior to the enzyme reaction and was used as an internal standard for quantification of the radioactivity after electrophoresis. Endonuclease V catalyzes the two-step reaction, but the $\beta$-elimination at the second step cannot be observed directly when the pyrimidine dimer substrates are used. Therefore, the reaction rates of the PD glycosylase step and the overall reaction were measured.

The substrates, [$^{32}$P]d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) and the $R_P$- and $S_P$-isomers of [$^{32}$P]d(GCACGT[PS]TGCACG)·d(CGTGCAACGTGC), were incubated with T4 endonuclease V, and aliquots were taken at intervals of 10 min. With and without the hot piperidine treatment for the PD glycosylase step and the overall reaction, respectively, the samples were mixed with the loading solution containing 90% formamide, and the products were separated by denaturing 20% PAGE. The products were quantified, and the sampling errors were corrected using the radioactivity of the 24-mer internal standard. From the plots of the time courses, shown in Figure 7, the rate constants of the PD glycosylase step and the overall reaction for each substrate were obtained (Table 2). There was no difference in these rate constants between the $R_P$- and $S_P$-isomers of the phosphorothioate substrate, although they were smaller than those for the parent duplex without the phosphate modification.

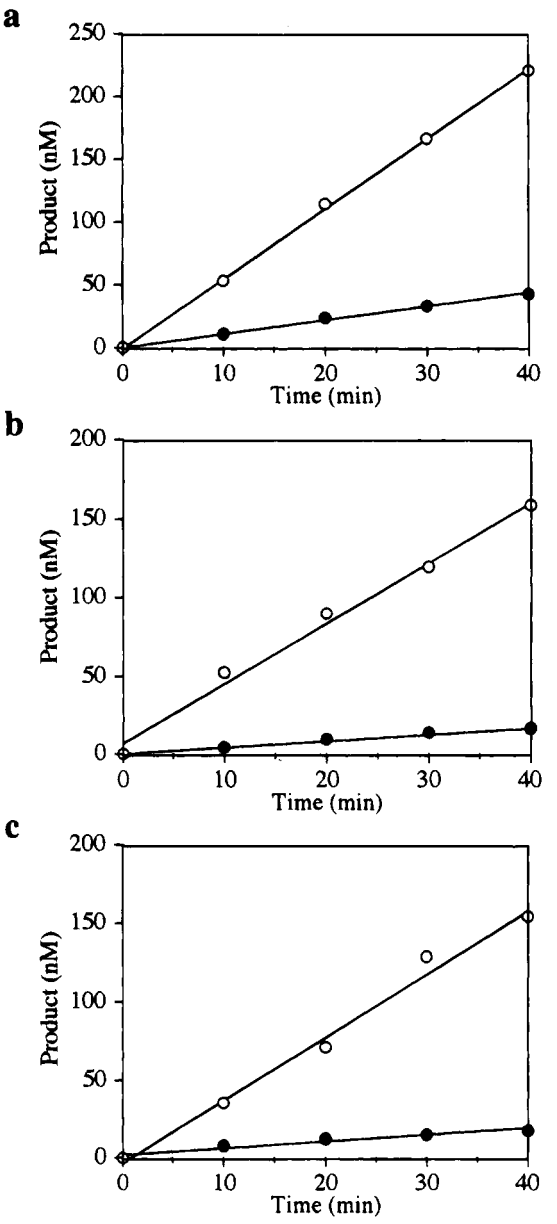


FIGURE 7: Time courses of the PD glycosylase (○) and overall (●) reactions. The substrates are d(GCACGT[]TGCACG)·d(CGTGCAACGTGC) (a) and the $R_P$ (b) and $S_P$ (c) isomers of d(GCACGT[PS]TGCACG)·d(CGTGCAACGTGC).

Table 2: Rate Constants ($min^{-1}$) Obtained under the Substrate-Saturation Conditions

| substrate | PD glycosylase | overall |
|---|---|---|
| d(GCACGT[]TGCACG)<br>d(CGTGCA ACGTGC) | 0.90 | 0.18 |
| $R_P$-d(GCACGT[PS]TGCACG)<br>d(CGTGCA ACGTGC) | 0.62 | 0.067 |
| $S_P$-d(GCACGT[PS]TGCACG)<br>d(CGTGCA ACGTGC) | 0.65 | 0.069 |

## DISCUSSION

Modified oligonucleotide duplexes were used as substrates to elucidate the reaction mechanism of endonuclease V from

bacteriophage T4. A mechanism in which a covalent intermediate is formed between the N-terminal amino group of the enzyme and the C1′ of the 5′-component of the pyrimidine dimer site (Figure 1) has been proposed for the PD glycosylase step (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993). (An in-line $S_N2$ reaction was proposed originally, but an $S_N1$ mechanism, in which the nucleophilic attack occurs after the glycosyl bond cleavage caused by the protonation to the C2 carbonyl group, is supposed to be proper.) A substrate analog containing a fluorine atom at the 2′-position of the thymine dimer site (Figure 2a) was used first to demonstrate the formation of this covalent intermediate. This is the first approach from the substrate side, while Schrock and Lloyd (1991, 1993) proved it by the modification of the enzyme. The fluorine atom withdraws the electron by the inductive effect and thus can stabilize the neighboring C−N bond against hydrolysis, when a covalent intermediate is formed. It should be noted that this duplex contained the hydrogen at the 2′-upper-position, which should be abstracted in the β-elimination at the second step of the endonuclease V reaction (Mazumder et al., 1989).

The strand cleavage by endonuclease V was not observed for the fluorine-containing duplex, although the enzyme binding to this duplex was shown by the glycosyl bond cleavage followed by the elimination with piperidine (Figure 4) and by the band-shift on a non-denaturing gel (Figure 6). Compared with the sugar-unmodified substrate, the glycosyl bond was cleaved more slowly (completely cleaved by the prolonged incubation). This result is attributed to the C2′-fluorine, which prevents the protonation to the C2 carbonyl of the pyrimidine dimer and destabilizes the abasic product with a positive charge at C1′ by the inductive effect. It has been described that substitution of an electron-withdrawing group at the C2-position of ribose stabilizes the cyclic hemiacetal form (Angyal, 1984, 1991). Since the β-elimination occurs only in the acyclic aldehyde form (or in the Schiff base-type intermediate in this case), it is suggested that the fluorine atom can inhibit the strand cleavage at the abasic site by stabilizing the inactive cyclic sugar moiety.

A covalent complex was detected by SDS−PAGE (Figure 5). Under reducing conditions, a result very similar to that of Dodson et al. (1993) was obtained. In the experiment using the fluorine-containing duplex, the migration of the shifted band was slightly slower than that of the reduced complex. The structural difference, i.e. cyclic and acyclic sugar moieties, may account for the mobility. The smearing observed between the two bands after a longer exposure suggested that the covalent bond formed between the enzyme and the fluorinated oligonucleotide was hydrolyzed during the electrophoresis. This hydrolysis may have occurred before the electrophoresis. In order to determine whether this incomplete band shift was caused by the hydrolysis of the C−N bond after the complete complex formation, as discussed above, or by the partial complex formation in the reaction mixture, the formation of the stabilized covalent intermediate was analyzed by other methods. The enzyme was inactivated by pretreatment with an equimolar amount of the fluorine-containing duplex (Figure 6a), and the complete band shift was observed by non-denaturing PAGE (Figure 6b). Although the enzyme−substrate complex without the covalent bond cannot be distinguished from the covalent complex on the non-denaturing gel, the inactivation result demonstrates that all the enzyme molecules were converted to the stable covalent complex with the fluorine-containing substrate analog in the reaction mixture.

The oligonucleotide duplex containing a fluorine atom at the 2′-position is a novel enzyme inhibitor. Osterman et al. (1988) used a DNA duplex containing 5-fluorocytosine as an inhibitor of *Hha*I methylase, in which the proton to be abstracted in the elimination reaction was replaced by a fluorine atom, but our duplex inhibited the enzyme reaction by stabilizing the covalent intermediate by the inductive effect of the fluorine atom, in spite of the existence of the corresponding proton. This kind of substrate analog is applicable to other enzymes such as *Escherichia coli* 8-oxoguanine-DNA glycosylase, for which a covalent intermediate at the 1′-position is proposed (Tchou et al., 1994).

Modified internucleotide linkages at the thymine dimer site have been used to study the substrate recognition and the catalytic reaction by T4 endonuclease V. In our previous work, the stereochemistry of the methylphosphonate linkage, in which one of the nonbridging oxygens of the phosphodiester was replaced by a methyl group, was used, and it was demonstrated that this enzyme interacted with its substrate in the minor groove of the duplex (Iwai et al., 1994). When the oligonucleotide duplex containing the $S_P$-methylphosphonate linkage at the thymine dimer site was treated with T4 endonuclease V, two β-elimination products were obtained. One was identical with the normal product, and the other was assumed to be the cis isomer of the α,β-unsaturated product, because a methylphosphonate was a better leaving group than a phosphate in the elimination reaction. Therefore, the methylphosphonate linkage is not a good analog to analyze the endonuclease V reaction. In the present study, the stereochemistry of the phosphorothioate linkage was applied to the analysis of the β-elimination reaction in the strand cleavage by T4 endonuclease V. The chiral phosphorothioates have been used in studies of the reaction and substrate recognition by *Eco*RI (Connolly et al., 1984a,b; Koziolkiewicz & Stec, 1992; Lesser et al., 1992), the mechanisms of catalytic RNAs (McSwiggen & Cech, 1989; Rajagopal et al., 1989; Moore & Sharp, 1993), and the stereochemical courses of pre-mRNA splicing and DNA strand transfer (Maschhoff & Padgett, 1993; Serre et al., 1993; Hanai & Wang, 1993). The difference in the duplex stability and the conformational change between the diastereomers of the modified oligonucleotides must be negligible for studies of enzyme−substrate interactions, but the phosphorothioate could be used successfully in these studies, because it was reported that the difference in the $T_m$ values was only 2.4 °C when two phosphorothioate linkages ($R_P$−$R_P$ and $S_P$−$S_P$) were incorporated into an 8-mer duplex (LaPlanche et al., 1986). For endonuclease V, it is important to use oligonucleotide duplexes containing a thymine dimer, which are the true substrates, while the mechanisms for the β-elimination were derived from experiments using duplexes containing an abasic site without a pyrimidine dimer (Mazumder et al., 1991; Hori et al., 1992).

The first results we obtained using the phosphorothioate substrates were the kinetic parameters of the PD glycosylase reaction by T4 endonuclease V, as shown in Table 1. The $K_m$ value for the $S_P$-isomer of the phosphorothioate-containing substrate was 13-fold larger than that for the $R_P$-isomer, although the difference in the $k_{cat}$ values was small. The difference in the affinities of the isomers for the enzyme was also shown by the $K_d$ values for the complexes. Since

the formation of a cis-syn thymine dimer induces only small distortions of the B-DNA structure, as revealed by NMR studies (Kemmink et al., 1987a,b; Taylor et al., 1990; Lee et al., 1994), the *pro*-$S_P$-oxygen of the internucleotide phosphodiester linkage at the thymine dimer site is accessible from the minor groove in which T4 endonuclease V makes contact with its substrate. In the $S_P$-phosphorothioate, this oxygen is replaced by a sulfur atom, and the negative charge is localized on the sulfur atom (Frey & Sammons, 1985). Consequently, the reduced affinity observed for the $S_P$-phosphorothioate substrate indicates the loss of a hydrogen-bonding interaction, which is formed between the enzyme and the *pro*-$S_P$-oxygen of the normal substrate or the oxygen atom of the $R_P$-phosphorothioate substrate.

Since a difference was found in the affinity of the enzyme for the duplexes containing the $R_P$- and $S_P$-phosphorothioate linkages, the $k_{cat}$ values in Table 1, which could be influenced by the affinity, could not be compared directly. In order to rule out this effect, reaction rates were measured under the saturation conditions. Since single-turnover experiments under the enzyme-saturation conditions were too difficult to carry out, we chose the substrate-saturation conditions. When the substrate concentration is much higher than the $K_m$ value, the concentration of the enzyme−substrate complex is equal to the total enzyme concentration, and the dissociation of the complex is negligible. Under these conditions, the catalytic rate constant includes the chemical step of the enzyme reaction and the subsequent product release. In our experiments, the $K_m$ values obtained earlier were used to set the substrate concentrations, and the substrates were treated with the enzyme at concentrations about 100-fold higher than each $K_m$. The reaction rates of the PD glycosylase step and the overall reaction (PD glycosylase and $\beta$-elimination) for the substrates containing either the phosphodiester or the phosphorothioate linkage at the thymine dimer site were measured, since the $\beta$-elimination step could not be analyzed directly. There was a difference in the rate constants of the overall reactions for the parent duplex and the phosphorothioate substrates (Table 2). Since the products are identical except that the sulfur atom is slightly larger than oxygen, this difference indicates that the rate-limiting step is not the product release, but the chemical reaction. The result that the reaction rate of the PD glycosylase step for each substrate was much higher than that of the overall reaction shows that the $\beta$-elimination is the rate-limiting step and that this step can be discussed by this approach.

There was no difference in the rate constants of the overall reactions for the $R_P$- and $S_P$-phosphorothioate substrates (Table 2). Since the rate constants of the first step, namely, the PD glycosylase reaction, were also the same, it is concluded that the reaction rates of the $\beta$-elimination are equal regardless of the absolute configuration of the cleaved phosphorothioate linkage. If the internucleotide phosphate residue 3′ to the abasic site produced by the PD glycosylase reaction abstracts the 2′-hydrogen, two stereochemical courses are assumed for the $\beta$-elimination reaction. One is the abstraction of the 2′-*pro*-*S*-hydrogen at the abasic site by the *pro*-$S_P$-oxygen of the phosphodiester, which has its position fixed by the hydrogen bond with the enzyme, and the other is that by the *pro*-$R_P$-oxygen, which is oriented for the reaction by the hydrogen bond formed between the other oxygen and the enzyme. Although no information is available about the distances between the 2′-*pro*-*S*-hydrogen and these oxygens in the abasic intermediate, a difference should be observed, in both cases, in the $\beta$-elimination rates for the diastereomers of the phosphorothioate-containing substrate, because of the different stereochemical arrangement and the localization of the negative charge on the sulfur atom. Consequently, the results obtained in this study indicate that some amino acid residue of T4 endonuclease V is the general-base catalyst in the $\beta$-elimination reaction. In both the PD glycosylase step and the overall reaction, the rate constants for the phosphorothioate substrates were smaller than those for the phosphodiester substrate. This reduction may be attributed to a slight conformational change of the substrate in the complex caused by the sulfur substitution. The amino acid residue responsible for the abstraction of the 2′-hydrogen could not be identified in this study, but a likely candidate is the glutamate at position 23 (Hori et al., 1992).

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