Endonuclease V from Bacteriophage T4 Interacts with Its Substrate in the Minor Groove†

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ABSTRACT: The binding of bacteriophage T4 endonuclease V to its substrate has been studied using synthetic oligodeoxyribonucleotide duplexes containing a cis–syn thymine dimer. Substrate analogues containing a methylphosphonate linkage with a defined configuration at the thymine dimer site were prepared, and the binding of the enzyme to each diastereomer was analyzed by the filter-binding method. The duplex containing a methylphosphonate with the S_P configuration formed a complex with the enzyme, although the dissociation constant for this substrate analogue was about 8 times larger than that for the 12-mer substrate containing a phosphodiester linkage at this site. In contrast, no binding was observed when a duplex containing the R_P-methylphosphonate linkage was used. The glycosyl bond of the thymine dimer in the S_P isomer was cleaved by the enzyme, while no incision was detected in the case of the R_P isomer, even after alkali treatment. Another substrate analogue containing a sulfur atom in place of the 3′-oxygen of the 5′-component at the thymine dimer site showed a reduced affinity for the enzyme. These results suggest that T4 endonuclease V interacts with its substrate in the minor groove. This mode of binding was confirmed by methylation protection experiments.

One of the well-characterized photolesions caused by ultraviolet (UV)[1] light irradiation is the cis–syn cyclobutane–pyrimidine dimer, which predominantly induces transitions from cytosine to thymine (Jiang & Taylor, 1993). In our previous study, transversions from thymine to adenine were observed most frequently on the 3′-side of the cis–syn thymine dimer (Kamiya et al., 1993). Endonuclease V from bacteriophage T4 initiates the repair of these lesions by the following process. This enzyme first binds to DNA in a nonspecific manner and scans along the DNA duplex processively to search for the target (Ganesan et al., 1986; Gruskin & Lloyd, 1986, 1988). Specific binding then occurs at the pyrimidine dimer. Incision of UV-irradiated DNA by T4 endonuclease V occurs by two combined activities. The first reaction catalyzed by this enzyme is the hydrolysis of the glycosyl bond of the pyrimidine dimer on the 5′-side of the dimer. A proposed mechanism suggests that a covalent intermediate between the N-terminal amino group of the enzyme and the C1′ of the 5′-deoxyribose residue at the pyrimidine dimer site is formed after protonation of the oxygen at C2 on the 5′-side of the dimer (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993). The second reaction is strand cleavage at the resultant apyrimidinic site by the β-elimination mechanism (Manoharan et al., 1988, 1989). This proceeds by a syn β-elimination involving abstraction of the 2′-*pro*-*S*-hydrogen and formation of a trans α,β-unsaturated product (Mazumder et al., 1989, 1991). A mechanism involving general-base catalysis by the phosphodiester group has been proposed (Mazumder et al., 1991; Schrock & Lloyd, 1991), while our results suggest that the glutamate residue at position 23 acts as a general base in the β-elimination reaction (Hori et al., 1992).

The X-ray crystal structure of T4 endonuclease V has been determined (Morikawa et al., 1992). This enzyme has an all-α structure with a positively charged internal curvature. The structure of DNA containing a cis–syn thymine dimer has been studied by nuclear magnetic resonance (NMR) spectroscopy (Kemmink et al., 1987a,b; Taylor et al., 1990; Lee et al., 1994). It was found that the formation of a dimer induced only small distortions in the B-DNA structure. At present, the mechanism for the recognition of the pyrimidine dimer by T4 endonuclease V has not been elucidated, although site-directed mutagenesis has been carried out at various positions in order to locate the amino acid residues responsible for substrate binding and catalytic reactions (Recinos & Lloyd, 1988; Stump & Lloyd, 1988; Dowd & Lloyd, 1989a,b, 1990; Ishida et al., 1990; Doi et al., 1992; Hori et al., 1992; Green et al., 1993).

In this article, we describe an investigation of the interactions between T4 endonuclease V and its substrate using chemically synthesized DNA duplexes containing a cis–syn thymine dimer. First, we modified the phosphate residue at the thymine dimer site. Oligonucleotides containing a methylphosphonate linkage at this site were prepared, and the two diastereomers were separated. An oligonucleotide containing a sulfur atom in place of the 3′-oxygen was also prepared. Modifications in the minor groove of the DNA duplexes resulted in either a reduction or a complete lack of enzyme binding. In order to confirm this minor groove binding, methylation protection experiments were carried out.

EXPERIMENTAL PROCEDURES

Materials. Organic chemicals were supplied by Wako Pure Chemical Industries. Reagents for the DNA synthesizer were purchased from Applied Biosystems. For the purification of dimethoxytritylated oligonucleotides by reversed-phase chromatography, Preparative C_{18} 125-Å (Millipore Corporation)

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[1] Abbreviations: UV, ultraviolet; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; TEAA, triethylammonium acetate; CPG, controlled-pore glass; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; $T_{PMe}T$, dithymidine methylphosphonate; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; SSC, 150 mM NaCl/15 mM trisodium citrate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; T[]T, cis–syn thymine dimer; $T_{[PMe]}T$, cis–syn thymine dimer with a methylphosphonate linkage at this site; $T_{[SP]}T$, cis–syn thymine dimer containing a sulfur atom in place of the 3′-oxygen at this site.

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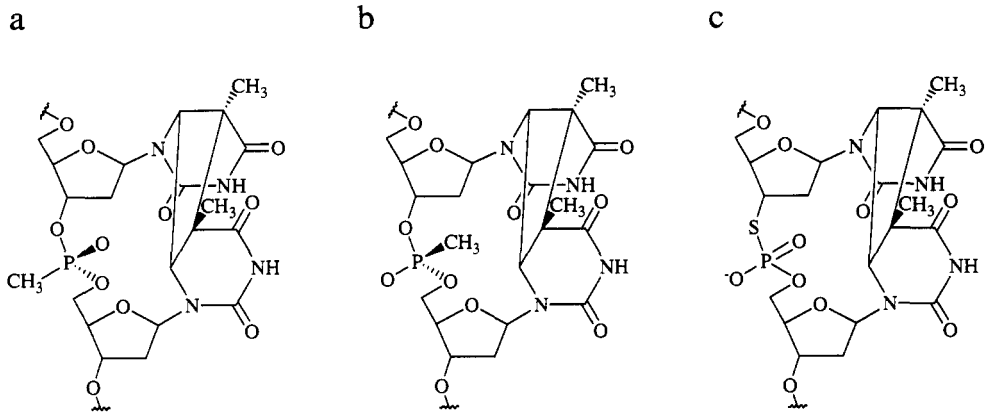

FIGURE 1: Structures of modified internucleotide linkages at the cis–syn thymine dimer: (a) R_P-methylphosphonate; (b) S_P-methylphosphonate; (c) phosphorothioate containing 3′-sulfur. These linkages were incorporated into the sequence of dGCACGT[]TGCACG.

was used. Purification of deprotected oligonucleotides was performed on a Gilson high-performance liquid chromatography (HPLC) instrument, using an Inertsil ODS-2 column (10 × 250 mm, GL Sciences Inc.) with a linear gradient of acetonitrile (HPLC grade, J. T. Baker Inc.) in 0.1 M triethylammonium acetate (TEAA, pH 7.0). The purity of the prepared oligonucleotides was analyzed on a Waters ALC/GPC 608 system or a Shimadzu LC-6A system, using both an Inertsil ODS-2 column (4.6 × 250 mm) with the above gradient and a TSK-GEL DEAE-2SW column (4.6 × 250 mm, Tosoh Corporation) with a linear gradient of ammonium formate in 20% aqueous acetonitrile. The photoproducts were separated on a μBondasphere 5-μm C_{18} 300-Å column (3.9 × 150 mm, Millipore). The absorbance of each oligonucleotide solution was measured at 260 nm on a Beckman DU-65 spectrophotometer, and the molarity was calculated as described (Cantor et al., 1970). [γ-^{32}P]ATP was obtained from New England Nuclear. T4 endonuclease V was produced and purified as described previously (Inaoka et al., 1989; Doi et al., 1992). T4 polynucleotide kinase was purchased from Takara Shuzo, nuclease P_1 was from Yamasa Corporation, and alkaline phosphatase (from calf intestine) was obtained from Boehringer Mannheim.

Preparation of Oligonucleotides. The chain assembly of oligonucleotides was carried out on either an Applied Biosystems 381A or 394 synthesizer. Unmodified oligodeoxyribonucleotides were synthesized using the reagents from Applied Biosystems and purified by HPLC. The dodecanucleotide containing a thymine dimer was prepared using a dimer coupling unit, as described previously (Murata et al., 1990). The preparation of the oligonucleotide containing a sulfur atom at the thymine dimer site is described elsewhere (Murata et al., 1992).

The dodecadeoxyribonucleotide containing a methylphosphonate linkage was synthesized on a 1.0-μmol scale using 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(*N*,*N*-diisopropyl)-methylphosphonamidite (BioGenex Laboratories). The reaction time for the coupling of the methylphosphonamidite was prolonged to 5 min, and the dimethoxytrityl group at the 5′-end was not removed. This oligonucleotide was cleaved from the controlled-pore glass (CPG) support with 30% aqueous ammonia at room temperature (for 1 h). After evaporation, a mixture of ethylenediamine, acetonitrile, and 95% ethanol (2:1:1, v/v/v) (2 mL) was added to the residue, and the resulting suspension was stirred for 6 h (Hogrefe et al., 1993). The solvent and ethylenediamine were removed by evaporation and coevaporation with ethanol. The residue was dissolved in 0.1 M TEAA, and the oligonucleotide with the dimethoxytrityl group at the 5′-end was purified by reversed-phase chromatography. This product was eluted with a gradient of acetonitrile (from 0 to 50%) in 0.1 M TEAA. After the fractions were concentrated and coevaporated with water, the dimethoxytrityl group was removed with 80% aqueous acetic acid, and the deprotected oligonucleotide was purified by reversed-phase HPLC. Two diastereomers were separated at this step (Figure 2a). About 15 A_{260} units of each diastereomer were obtained starting from 1.0 μmol of deoxycytidine–CPG. The formation of the thymine dimer in each oligonucleotide was carried out by UV irradiation, as described previously (Inaoka et al., 1989).

Determination of the Absolute Configuration of the Methylphosphonate. Dithymidine methylphosphonate with 4,4′-dimethoxytrityl and benzoyl groups, for the protection of the 5′- and 3′-hydroxyl functions, respectively, was prepared as described (Miller et al., 1983). The two diastereomers were separated by chromatography on silica gel with a solvent system of dichloromethane/acetone. Aliquots (30 mg) of these compounds were dissolved in $CDCl_3$/pyridine-d_5 (9:1, v/v, 0.5 mL), and ROESY spectra were recorded with a CW pulse for a spin–lock of 400 ms on a Bruker AMX600 spectrometer. After deprotection with ethanolic ammonia and then 80% aqueous acetic acid, followed by reversed-phase HPLC purification, the circular dichroism (CD) spectra of each isomer in 1 M NaCl, 10 mM sodium phosphate, and 60 μM EDTA (pH 7.0) were recorded on a Jasco J-720 spectropolarimeter. From these spectra, the absolute configuration of the deprotected dithymidine methylphosphonate with a shorter retention time by reversed-phase HPLC was assigned as R_P, and that with a longer retention time was assigned as S_P.

The two diastereomers of dGCACGT$_{PMe}$TGCACG were degraded to their nucleoside components, and their absolute configulations were determined as follows. The oligonucleotides (2.0 A_{260} units) were dissolved in water (40 μL) and 0.1 M ammonium acetate (pH 5.3, 50 μL), and nuclease P_1 (1 mg/mL, 10 μL) was added. The mixtures were incubated at 50 °C for 10 h and then diluted with water (347 μL) and 0.5 M Tris-HCl (pH 9.0, 50 μL). Alkaline phosphatase (1 unit/μL, 3 μL) was added, and the mixtures were incubated at 37 °C for 14 h. After ethanol (1.5 mL) was added and the mixtures were incubated at −20 °C for 2 h, the proteins were pelleted by centrifugation, and the supernatants were concentrated *in vacuo*. The residues were dissolved in 50% aqueous acetonitrile (500 μL) and filtered through a membrane filter (Millipore Columngard-LCR$_4$). Aliquots (5 μL) of these solutions were analyzed by reversed-phase HPLC (Figure 3).

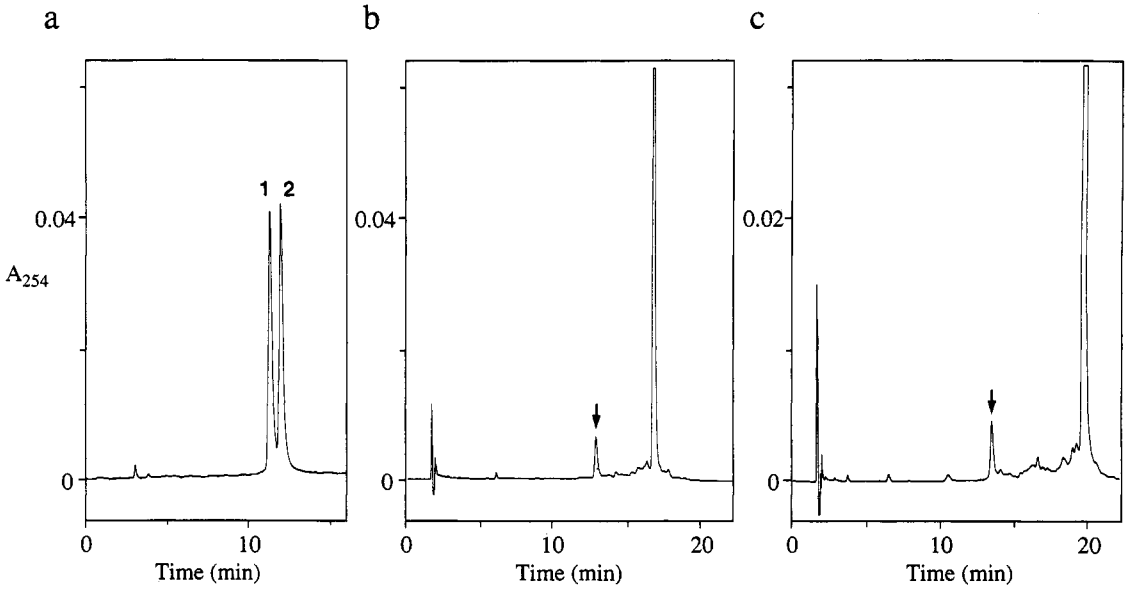

FIGURE 2: (a) Analysis of dGCACGT$_{PMe}$TGCACG by reversed-phase HPLC. Elution was performed using a linear gradient of acetonitrile (from 9% to 13% over 20 min) in 0.1 M TEAA. The two diastereomers (peaks 1 and 2) were separated under the same conditions on a preparative scale. (b and c) HPLC analysis of the irradiated products of isomer 1 (peak 1 in a) and isomer 2 (peak 2), respectively. Each peak indicated by an arrow contained the cyclobutane–thymine dimer.

The authentic R_P- and S_P-dithymidine methylphosphonates were eluted under the same conditions.

Filter-Binding Experiments. The oligonucleotides containing a thymine dimer (100 pmol) were labeled using [γ-^{32}P]-ATP (1.5 × 10^6 cpm) and T4 polynucleotide kinase (10 units) in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 10 μM ATP (10 μL). After incubation at 37 °C for 1 h, the mixtures were diluted with water to 200 μL and passed through a Nensorb 20 column (DuPont). After concentration, the labeled oligonucleotides (60 pmol) were mixed with the complementary strand (120 pmol) in water (60 μL), and the solutions were incubated at 40 °C for 3 min and then cooled to 4 °C.

The binding reactions (50 μL) contained 20 nM ^{32}P-labeled oligonucleotide duplex (approximately 15 000 cpm) and varying concentrations of T4 endonuclease V in 9 mM Tris-HCl (pH 8.0), 90 mM NaCl, 9 mM EDTA, and 10% ethylene glycol. After incubation on ice for 1 min, the mixtures were diluted with 0.6 mL of 14× SSC (2.1 M NaCl and 0.21 M trisodium citrate) (Seawell et al., 1980) and passed through a 25-mm cellulose nitrate membrane filter with a pore size of 0.45 μm, which had been prewashed twice with 0.6 mL of ice-cold 14× SSC. The filters were washed with 0.6 mL of ice-cold 14× SSC and dried in a 50 °C oven. The radioactivity retained on the filters was quantified on a FUJIX BAS2000 bio-imaging analyzer (Fuji Photo Film).

Analysis of Glycosyl Bond Cleavage. The ^{32}P-labeled oligonucleotide duplexes (2 pmol, about 3000 cpm) were mixed with T4 endonuclease V in a solution (20 μL) containing 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA, and 100 mM NaCl and incubated at 30 °C for 12 min. For the hot piperidine treatment, 0.15 M aqueous piperidine (40 μL) was added, and the mixtures were heated at 95 °C for 30 min. The piperidine was removed by evaporation and coevaporation with water. The products were separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

Methylation Protection. The 30-mers (dGACTGTCG-GTACGTAG-AA-GATGCGTACGTG and dGACTGTCG-GTACGTAG-GG-GATGCGTACGTG) were labeled as described above and annealed to the oligonucleotide containing a thymine dimer (dCACGTACGCATCT[]TCTACGTAC-CGACAGTC). These duplexes (5 pmol) were dissolved in a solution (200 μL) containing 50 mM sodium cacodylate (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, and 100 mM NaCl and mixed with T4 endonuclease V. The mixtures were kept on ice for 1 min, and then dimethyl sulfate (12 μL) was added. After incubation on ice for 1 min, a solution (50 μL) containing 1.0 M Tris-HCl (pH 7.5), 1.0 M mercaptoethanol, 1.5 M sodium acetate, and 1 mM EDTA was added. After the addition of a 10 mg/mL tRNA solution (0.5 μL) and ethanol precipitation, the precipitates were dissolved in a buffer (20 μL) containing 10 mM sodium phosphate (pH 7.0) and 1 mM EDTA and heated at 90 °C for 15 min. A 1.0 M NaOH solution (2 μL) was added, and the mixtures were heated again at 90 °C for 30 min. The products were separated by 20% denaturing polyacrylamide gel electrophoresis (PAGE).

RESULTS

Preparation of Modified Oligonucleotides Containing a Thymine Dimer. Two kinds of modified oligodeoxyribonucleotides were synthesized to investigate the interactions between T4 endonuclease V and the phosphate residue at the cyclobutane–pyrimidine dimer. One was a 12-mer containing a methylphosphonate linkage at the cis–syn thymine dimer site (dGCACGT[$_{PMe}$]TGCACG) (Figure 1a,b). Two diastereomers are generated by this modification due to the chiral phosphorus, and only the oxygen atom, and not the methyl group, can form a cation–dipole interaction with the enzyme or be a proton acceptor in hydrogen bonding. The oxygen atom of the R_P isomer (Figure 1a) resides in the major groove of B-form DNA, and that of the S_P isomer (Figure 1b) lies in the minor groove.

First, a 12-mer containing a methylphosphonate linkage between two adjacent thymidines (dGCACGT$_{PMe}$TGCACG) was synthesized using 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(*N*,*N*-diisopropyl)methylphosphonamidite. Deprotection of this oligonucleotide was carried out as reported recently (Hogrefe et al., 1993), and the product with a dimethoxytrityl group at the 5′-end was purified by reversed-phase chromatography. After detritylation, the two diastereomers were separated by reversed-phase HPLC, as shown in Figure 2a.

The thymine dimer was formed in each oligonucleotide by UV irradiation using a mercury light filtered with a colored glass and a $NiSO_4$ solution, as described previously (Inaoka et al., 1989). After irradiation for 3 h, the photoproducts were obtained, as shown in Figure 2b,c. The products indicated by an arrow were reversed to each starting material by UV irradiation (data not shown). This photoreversion is evidence of the formation of the cyclobutane–thymine dimer (Taylor et al., 1987). The yield of the thymine dimer oligonucleotide from isomer 1, corresponding to peak 1 in Figure 2a, was 3.1%, and that from isomer 2 was 1.5%. Longer exposure to UV light resulted in more byproduct formation.

The other modified oligonucleotide was a 12-mer containing a sulfur atom in place of the 3′-oxygen (dGCACGT[$_{SP}$]-TGCACG) (Figure 1c). This position is accessible from the minor groove side, but not from the major groove side. The synthetic procedure for this oligonucleotide was reported elsewhere (Murata et al., 1992). As a control, a 12-mer with a normal phosphodiester linkage at this site (dGCACGT[]-TGCACG) was prepared (Murata et al., 1990) using a thymine dimer phosphoramidite unit, as reported by Taylor et al. (1987). A 30-mer containing a thymine dimer (dCACGTACGCATCT[]TCTACGTACCGACAGTC) for the methylation protection experiments was also prepared in the same way.

The 12-mers containing a thymine dimer were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and annealed to the complementary strand (dCGTGCAACGTGC). The 30-mer for methylation protection was annealed to either the 5′-^{32}P-labeled complementary sequence (dGACTGTCGG-TACGTAGAAGATGCGTACGTG) or a ^{32}P-labeled oligonucleotide with a GG sequence opposite the thymine dimer (dGACTGTCGGTACGTAG-GG-GATGCGTACGTG).

Determination of the Absolute Configuration of the Methylphosphonate. First, dithymidine methylphosphonate ($T_{PMe}T$) was prepared, and the two isomers were separated by chromatography on silica gel. These dimers had 4,4′-dimethoxytrityl and benzoyl groups as protecting groups at the 5′- and 3′-ends, respectively. The ROESY spectra of these compounds were recorded. Löschner and Engels (1990) reported that an ROE from the methyl group to the H4′ of the 5′-component was observed only in the R_P isomer of the protected dithymidine methylphosphonate. In our ROESY spectra, the isomer with the larger R_f value by silica gel TLC ($T_{PMe}T1$) gave intense crosspeaks from the methyl group to the H4′ of the 5′-thymidine, as well as to the H3′ of the 5′-thymidine and the H5′ of the 3′-thymidine, but these crosspeaks were weaker in the case of the isomer with the smaller R_f value ($T_{PMe}T2$) (data not shown). After deprotection, the CD spectra were recorded. Lebedev et al. (1993) noted that the magnitude of the molecular ellipticity was higher for the R_P isomer than for the S_P. In our CD profiles, the difference between the 280- and 250-nm bands of $T_{PMe}T1$ was 2.6 times larger than that of $T_{PMe}T2$. Both results show that $T_{PMe}T1$ and $T_{PMe}T2$ contain the methylphosphonate linkages with the R_P and S_P configurations, respectively. The relationship between the order of elution from a silica gel column and the configurations was the same as that reported previously (Löschner & Engels, 1990; Lebedev et al., 1993).

The absolute configuration of the methylphosphonate in each separated isomer of dGCACGT$_{PMe}$TGCACG was determined using these dithymidine methylphosphonates with defined configurations. The 12-mers were degraded with nuclease P_1 and alkaline phosphatase to their nucleoside components, and the products were separated by reversed-phase HPLC (Figure 3). Since the methylphosphonate linkage was not cleaved by nuclease P_1, four peaks, corresponding to dC, dG, dA, and $T_{PMe}T$, were obtained. The retention times of the deprotected $T_{PMe}T1$ and $T_{PMe}T2$ isomers under the same conditions were 29.1 and 30.3 min, respectively, which coincided with those of $T_{PMe}T$ derived from isomer 1 (peak 1 in Figure 2a) and isomer 2 (peak 2), respectively. This identity was confirmed by coinjection of the authentic dimers with the degraded products. Consequently, the absolute configuration of the methylphosphonate linkage in isomer 1 was R_P and that in isomer 2 was S_P.

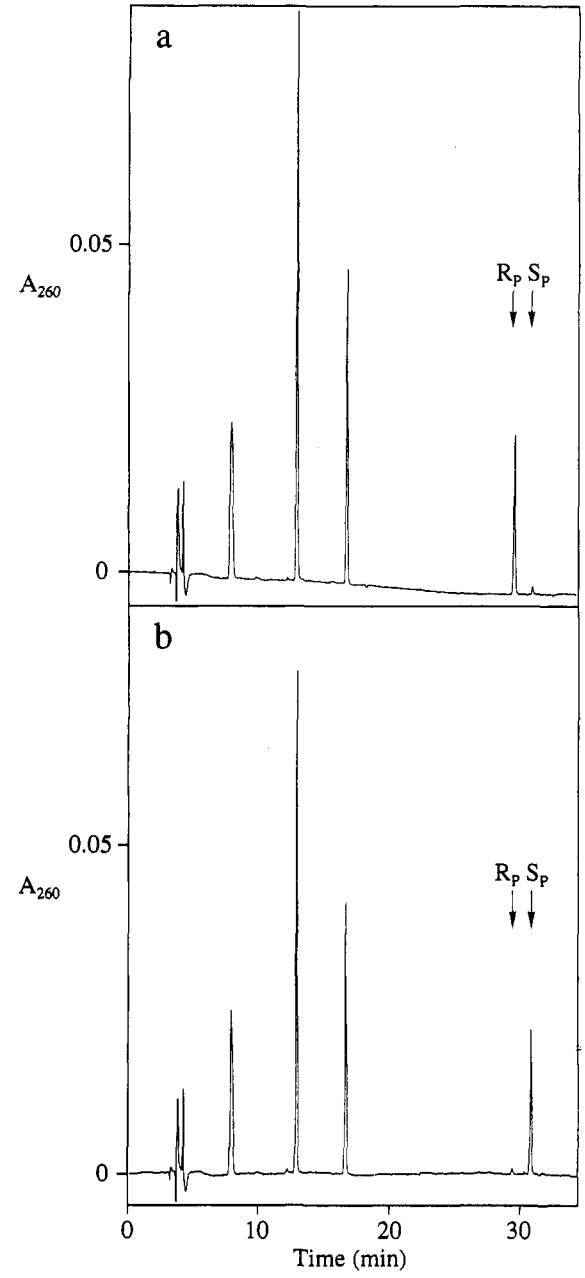

FIGURE 3: Analysis of the degraded products of dGCACGT$_{PMe}$-TGCACG by reversed-phase HPLC: (a) isomer 1 (peak 1 in Figure 2a); (b) isomer 2 (peak 2). Elution was performed using a linear gradient of acetonitrile (from 0% to 20% over 40 min) in 0.1 M TEAA. The retention times of the R_P- and S_P-dithymidine methylphosphonates are shown by arrows.

Binding of T4 Endonuclease V to the Thymine Dimer DNA Containing a Methylphosphonate Linkage. Filter-binding experiments (Seawell et al., 1980; Inaoka et al., 1989) were carried out to show the affinity of T4 endonuclease V for the ^{32}P-labeled duplexes containing a methylphosphonate linkage

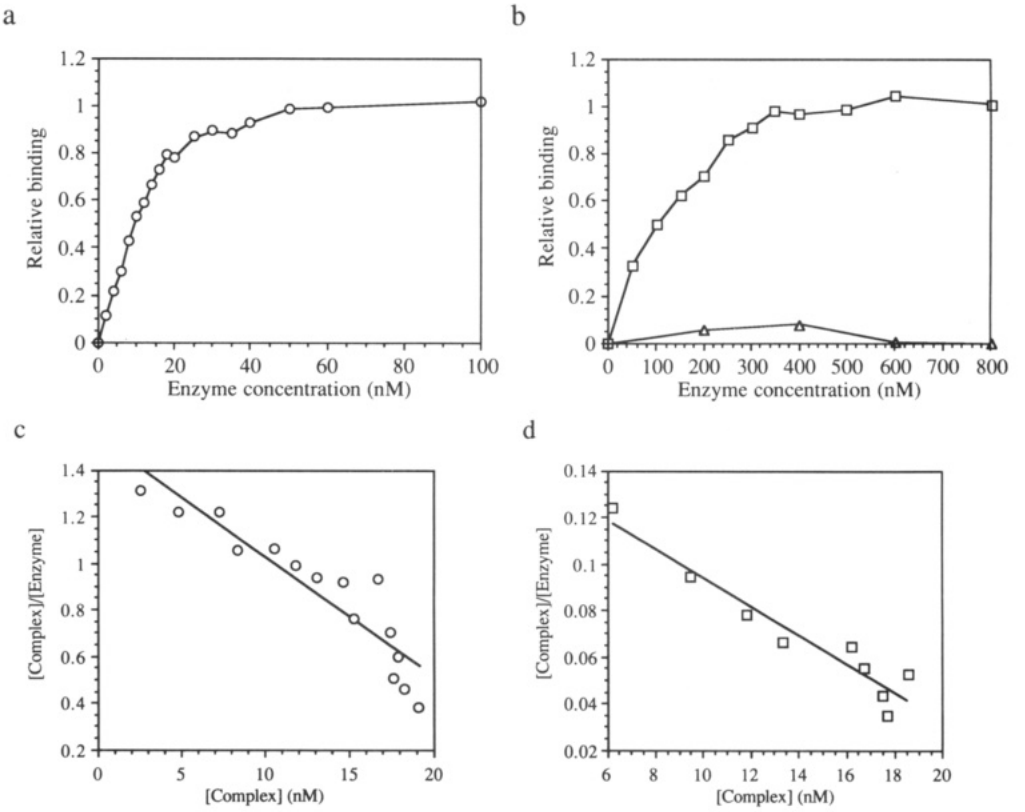

FIGURE 4: (a and b) Binding of T4 endonuclease V to dGCACGT[]TGCACG·dCGTGCAACGTGC (○) and the R_P (△) and S_P (□) isomers of dGCACGT[$_{PMe}$]TGCACG·dCGTGCAACGTGC. The data obtained from the filter-binding experiments were normalized. (c and d) Scatchard plots for binding of the enzyme to dGCACGT[]TGCACG·dCGTGCAACGTGC and to the S_P isomer of dGCACGT[$_{PMe}$]-TGCACG·dCGTGCAACGTGC, respectively.

at the thymine dimer site. When the normal 12-mer duplex containing a thymine dimer (dGCACGT[]TGCACG·dCGTG-CAACGTGC) was used, a binding curve saturated at an enzyme concentration of 50 nM was obtained (Figure 4a). By Scatchard analysis of these data, the dissociation constant (K_d) for this complex was calculated to be 2.0×10^{-8} M (Figure 4c). Compared to this result, a higher protein concentration was required for saturation in the case of the 12-mer duplex containing a methylphosphonate linkage with the S_P configuration (Figure 4b), and the K_d value was 1.6×10^{-7} M (Figure 4d). The R_P isomer showed almost no binding to T4 endonuclease V under the same conditions (Figure 4b).

Enzymatic Cleavage of the Glycosyl Bond in the Methylphosphonate-Containing DNA. Binding of T4 endonuclease V to the methylphosphonate-containing duplexes was also tested by analyzing the enzymatic cleavage of the glycosyl bond in these substrate analogues. The normal 12-mer duplex containing a thymine dimer and the methylphosphonate-containing duplexes were incubated with T4 endonuclease V, and the chain cleavage with and without piperidine treatment was analyzed by 20% denaturing PAGE (Figure 5). In the case of the S_P-methylphosphonate 12-mer, the glycosyl bond of the thymine dimer was cleaved (lanes 17–24), although a higher enzyme concentration was required for this substrate analogue than for the normal 12-mer duplex (lanes 1–8). Two bands were obtained without piperidine treatment in the case of the S_P-methylphosphonate duplex (lanes 19 and 20). These could be cis and trans isomers of the α,β-unsaturated product (Smith & Taylor, 1993). By treatment with hot piperidine, chain cleavage proceeded further to the δ-elimination product (lanes 6–8, 23, and 24). On the other hand, the substrate analogue containing a methylphosphonate linkage with the R_P configuration was not cleaved, even by the hot piperidine treatment (lanes 9–16).

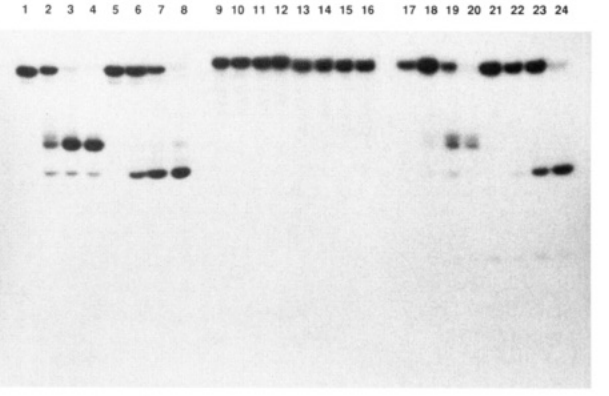

FIGURE 5: T4 endonuclease V cleavage of dGCACGT[]TGCACG·dCGTGCAACGTGC (lanes 1–8) and the R_P (lanes 9–16) and S_P (lanes 17–24) isomers of dGCACGT[$_{PMe}$]TGCACG·dCGTGC-AACGTGC. Lanes 1–4: without piperidine treatment, at enzyme concentrations of 0, 2.5, 5, and 15 nM, respectively. Lanes 5–8: with piperidine treatment at the same enzyme concentrations as lanes 1–4. Lanes 9–12 and 17–20: without piperidine treatment at enzyme concentrations of 0, 30, 60, and 200 nM, respectively. Lanes 13–16 and 21–24: with piperidine treatment at the same enzyme concentrations as lanes 9–12 and 17–20.

Binding of T4 Endonuclease V to the Thymine Dimer DNA Containing a 3′-Sulfur. Filter-binding experiments and enzymatic cleavage of the glycosyl bond were carried out using the ^{32}P-labeled 12-mer duplex containing a sulfur atom in place of the 3′-oxygen of the 5′-component at the thymine dimer (dGCACGT[$_{SP}$]TGCACG·dCGTGCAACGTGC). The amount of radioactivity retained on the filters was dependent on the protein concentration (data not shown), and the K_d value of 2.7×10^{-7} M was obtained by Scatchard analysis. Incubation of this duplex with T4 endonuclease V, followed by piperidine treatment, resulted in chain cleavage, although

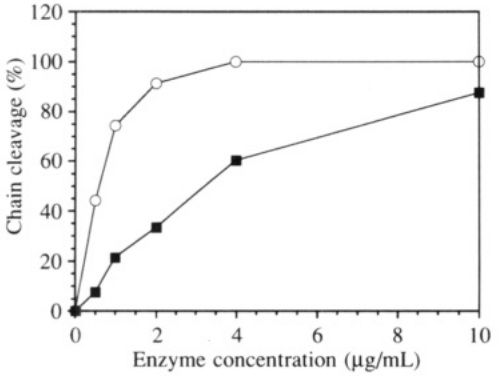

FIGURE 6: Chain cleavage of dGCACGT[]TGCACG·dCGTGCAACGTGC (○) and dGCACGT[SP]TGCACG·dCGTGCAACGTGC (■) by T4 endonuclease V.

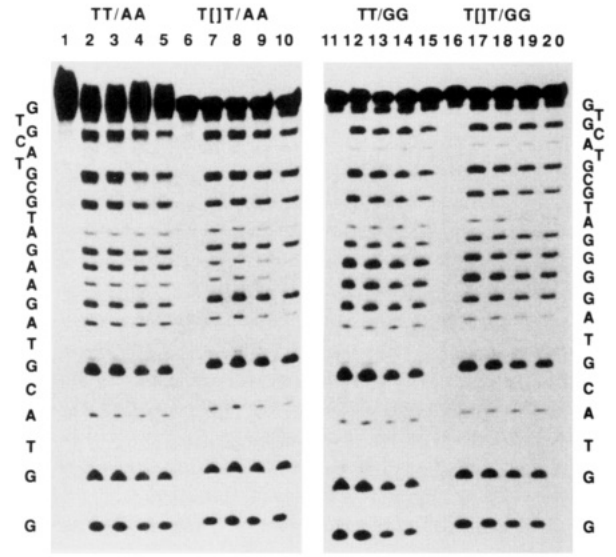

FIGURE 7: Methylation protection using dGACTGTCGGTACGTAGAAGATGCGTACGTG (lanes 1–10) or dGACTGTCGGTACGTAGGGATGCGTACGTG (lanes 11–20) as the complementary strand to dCACGTACGCATCTTCTACGTACCGACAGTC (lanes 1–5 and 11–15) or dCACGTACGCATCT[]TCTACGTACCGACAGTC (lanes 6–10 and 16–20). Lanes 1, 6, 11, and 16: without dimethyl sulfate or T4 endonuclease V. Lanes 2–5, 7–10, 12–15, and 17–20: treated with dimethyl sulfate at enzyme concentrations of 0, 0.2, 1.0, and 2.0 μM, respectively.

the rate of cleavage was lower than that for the normal 12-mer substrate (Figure 6).

Methylation Protection. After the enzyme was bound to DNA duplexes containing a thymine dimer, these complexes were methylated with dimethyl sulfate, and the positions where protection occurred by complex formation were determined. In this reaction, methylation occurs at the N3 of adenines and the N7 of guanines, which reside in the minor groove and in the major groove of the duplex, respectively. These methylated bases were removed by heating under neutral conditions, and the chains were cleaved at the resultant abasic sites by alkali treatment. Since the thymine dimer strand could be cleaved by complex formation and the following alkali treatment, the strands complementary to the thymine dimer sequence were labeled in these experiments, and the products were separated by 20% denaturing PAGE. When a complementary 30-mer duplex containing a thymine dimer (dCACGTACGCATCT[]-TCTACGTACCGACAGTC·[^{32}P]dGACTGTCGGT-ACGTAGAAGATGCGTACGTG) was used, the magnitude of the methylation of the adenines at positions 15, 17, 18, and 20 (counting from the 5′-end) was decreased, depending on the protein concentration, whereas there was no protection in the methylation of the guanines (Figure 7, lanes 6–10). Without the thymine dimer, only protection due to nonspecific protein binding was observed (Figure 7, lanes 1–5). When the two adenines opposite the thymine dimer were changed to guanines using [^{32}P]dGACTGTCGGTACGTAG-GG-GATGCGTACGTG as the complementary strand, these two guanines in the specific complex were methylated, while the methylation of the adenines at positions 15 and 20 was blocked (Figure 7, lanes 16–20).

DISCUSSION

NMR studies on the tertiary structure of oligonucleotide duplexes containing a cis–syn thymine dimer revealed that the dimer formation induced only small distortions of the B-DNA structure and that all base pairs, including the two AT pairs at the thymine dimer site, were formed (Kemmink et al., 1987a,b; Taylor et al., 1990; Lee et al., 1994). These results are in agreement with those obtained in a study of the energetics of oligonucleotide duplexes containing a thymine dimer (Rao et al., 1984) and in experiments using A-tract sequences to calculate the bending angle induced by photodimerization (Wang & Taylor, 1991). Our present study is based on these structural characteristics of the DNA duplex containing a thymine dimer.

In the experiments using DNA duplexes containing a methylphosphonate linkage, structural deviations from the parent compounds containing a phosphodiester at this site must be considered. The base stacking conformations were studied using dinucleoside methylphosphonates, and it was found that the base stacking of the R_P isomers was similar to that of the parent dinucleoside monophosphates and that the S_P isomers had conformations with less stacking (Miller et al., 1979; Kan et al., 1980; Löschner & Engels, 1990; Lebedev et al., 1993). When methylphosphonates were incorporated into the oligonucleotide duplexes, the duplexes containing the R_P-methylphosphonate linkage gave a melting curve similar to that of the parent duplex, whereas the S_P-methylphosphonate reduced duplex stability (Bower et al., 1987). In our study, T4 endonuclease V bound to the duplex containing the S_P-methylphosphonate linkage, which could destabilize the duplex to a greater extent than the R_P isomer. Therefore, it is obvious that the loss of enzyme binding to the duplex containing the R_P-methylphosphonate linkage was not due to dissociation or distortion of the duplex. The K_d value for the complex containing the S_P-methylphosphonate was 8 times larger than that for the complex with the parent duplex. This difference suggests that an ionic interaction is formed between T4 endonuclease V and the phosphate residue at this site.

Methylphosphonates with defined absolute configurations have been used to investigate their influence on duplex destabilization (Bower et al., 1987), but very few applications to protein–nucleic acid interactions have been reported. A mixture of diastereomers of the methylphosphonate linkage was used to probe the interactions between human immunodeficiency virus type-1 *tat* protein and TAR RNA (Hamy et al., 1993). Separated isomers were used to study the binding of *lac* repressor to *lac* operator, but the absolute configurations were not determined (Noble et al., 1984). Our results prove that the stereochemistry of methylphosphonates can be used to investigate protein–nucleic acid interactions.

In the experiments using an oligonucleotide duplex in which a sulfur atom was substituted for the 3′-oxygen at the thymine dimer site, the K_d value was more than 10 times larger than that for the complex with the parent duplex containing a normal phosphodiester linkage at this site. The differences between

this substrate analogue and the parent duplex are the size of the atom at this position and its ability to be a proton acceptor in hydrogen bonding with the enzyme. When a duplex containing a phosphorodithioate linkage at the thymine dimer site was used as a substrate analogue, the K_d value was almost the same as that for the normal substrate (Murata et al., 1990). Even a change in the sugar puckering, by the incorporation of 2′-fluorothymidine as the 5′-component of the dimer, did not affect the affinity (T. Murata and S. Iwai, unpublished result). Therefore, the larger K_d value was attributed to a loss of hydrogen bonding to this position due to the sulfur substitution.

All of the results described above show that T4 endonuclease V interacts with its substrate in the minor groove. Modifications in the minor groove of the DNA duplex, such as methyl substitution for the *pro-S*-oxygen of the phosphodiester linkage or sulfur substitution for the 3′-oxygen at the thymine dimer site, reduced its affinity for the enzyme. In the case of the R_P-methylphosphonate, binding of the enzyme was completely lost. On the other hand, modification of the *pro-R*-oxygen in the major groove induced a minor destabilization of the complex, which was probably caused by a shift from a cation–anion interaction to a cation–dipole interaction. The contacts in the minor groove were further substantiated by the methylation protection experiments (Johnson et al., 1978; Johnsrud, 1978). When the complexes of T4 endonuclease V with its substrate were treated with dimethyl sulfate, methylation occurred at the N7 of the guanines in the major groove, even in the presence of the enzyme, and was blocked at the N3 of the adenines in the minor groove. Since binding of T4 endonuclease V is specific to the cis–syn pyrimidine dimers, the most probable discrimination sites in the pyrimidine dimer are the two carbonyl groups at the C2 positions, which have fixed angles in the minor groove and are more polarized by the dimer formation. Possible interactions include hydrogen bonds between the enzyme and these two carbonyl groups, as well as hydrogen bonding to the 3′-oxygen and an ionic interaction at the *pro-S*-oxygen of the phosphodiester linkage at the pyrimidine dimer site.

Three bands were obtained when the substrate analogue containing the S_P-methylphosphonate linkage was treated with T4 endonuclease V (Figure 5, lanes 18–20). This enzyme cleaved the glycosyl bond of the thymine dimer on its 5′-side after binding to this substrate analogue, and chain cleavage occurred by an elimination reaction at the resultant abasic site. The faint band with the largest mobility is the δ-elimination product, corresponding to that obtained by the hot piperidine treatment (lanes 22–24) (Mazumder et al., 1991; Smith & Taylor, 1993). The lower major band corresponds to the product of the enzymatic chain cleavage of the normal substrate (lanes 2–4). In this reaction, the trans α,β-unsaturated aldehyde is produced by a syn β-elimination reaction (Mazumder et al., 1989). Since the methylphosphonate is a good leaving group in elimination reactions, the third band migrating most slowly could be assigned to the cis α,β-unsaturated aldehyde (or its hemiacetal form) produced by a chemical β-elimination. This nonenzymatic elimination reaction was described by Mazumder et al. (1991), and Smith and Taylor (1993) also discussed the cis products.

Our results for minor groove binding are congruent with the proposed mechanism: the protonation of the carbonyl group at the C2 position and the formation of the covalent enzyme–substrate intermediate at the C1′ position on the 5′-side of the dimer occur prior to glycosyl bond cleavage (Schrock & Lloyd, 1991, 1993; Dodson et al., 1993), because these positions are accessible from the minor groove side. Our observation that a substrate analogue containing a methylphosphonate linkage at the thymine dimer site was incised enzymatically suggests that the second reaction (β-elimination at the abasic site) is caused by a basic amino acid residue in the enzyme, not by the phosphodiester group itself, although a detailed kinetic analysis of this reaction could not be performed in this study. Elucidation of the catalytic mechanisms using other kinds of modified oligonucleotides is in progress and will be published elsewhere.

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