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Cleavage of dT_8 and dT_8 Phosphorothioyl Analogues by *Escherichia coli* DNA Topoisomerase I: Product and Rate Analysis

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ABSTRACT: *Escherichia coli* DNA topoisomerase I catalyzes the cleavage of short, single-stranded oligodeoxynucleotides with dT_8 as the shortest cleavable oligo(thymidylic acid). The 5′-^{32}P-labeled products formed from the cleavage of [5′-^{32}P]dT_8 are dT_5, dT_4, and dT_3 with over 70% of the substrate cleaved to dT_4. Mg(II) ions affect this product distribution by increasing the percentage of dT_4 formed. The substitution of a sulfur atom for a nonbridging oxygen atom in a phosphodiester linkage yields oligodeoxynucleotide phosphorothioyl (PS) analogues. The epimers of the analogues were separated, and the position and stereochemistry of the phosphorothiodiester bond were determined. Topoisomerase I is stereospecific in its reactivity toward these analogues. With the oligodeoxynucleotide PS analogue substrates, the rate of cleavage, the stereospecificity, and the product distribution depend upon the position and the stereochemistry of the phosphorothiodiester linkage.

Escherichia coli DNA topoisomerase I catalyzes the relaxation of negatively supercoiled DNA. After one relaxation event, the linking number of the supercoiled DNA substrate has increased by 1 (Brown & Cozzarelli, 1981). Evidence suggests that the enzyme breaks one strand of the DNA duplex in a single-stranded region (Wang, 1971), actively or passively passes the other intact strand through the break, and finally rejoins the broken strand (Gellert, 1981).

A two-domain model depicting the interaction of the bacterial enzyme with DNA has been proposed (Brown & Cozzarelli, 1981; Kirkegaard et al., 1984; Tse-Dinh, 1986). Evidence for a covalent complex via a protein tyrosine residue–DNA covalent linkage suggests a covalent binding domain (Tse et al., 1980). In this model then, a covalent binding domain contains a nucleophilic tyrosine residue at its active site which can displace the 5′ region of the DNA through a transesterification reaction during the strand breakage step. A covalent bond between the tyrosine and the 5′-phosphate of the 3′ region is formed. The model holds that a noncovalent binding domain interact with the broken DNA strand, if not both DNA strands, during strand breakage and passage. This domain may also facilitate re-formation of the DNA phosphodiester bond after strand passage by positioning the 3′-OH group of the 5′ region of DNA during the rejoining step.

The mechanism of *Escherichia coli* topoisomerase I catalyzed cleavage of oligodeoxynucleotides, or oligomers, is believed to mimic the covalent complexation between the protein's covalent binding domain and negatively supercoiled DNA. However, with a small, single-stranded oligomer substrate, the strand 5′ to the point of transesterification is released after cleavage (Tse-Dinh, 1986; Tse-Dinh et al., 1983). The dissociation of the substrate's 5′ region is probably due to its short length which does not allow for sufficient interaction with the enzyme's noncovalent binding domain. When long single-stranded DNA polymers are present, interaction with the noncovalent domain may come into play (Tse-Dinh, 1986). One major difference between the enzyme's reaction with supercoiled DNA and that with the oligodeoxynucleotides is that enzyme turnover with the latter is defined by the hydrolysis of the covalent complex (Tse-Dinh, 1986).

E. coli topoisomerase I cleaves dT_8 oligomers predominately between the fourth and fifth nucleotide (Tse-Dinh et al., 1983). The enzyme probably has substrate recognition sites both 5′ and 3′ to the actual site of cleavage. Toward understanding the enzyme's recognition criteria and the relative importance of these recognition sites, we analyzed the products formed and the rates of product formation from the enzymatic cleavage of dT_8 and phosphorothioyl analogues in both the presence and absence of divalent Mg ions. Three internucleotidyl phosphorothioyl substitutions were made—directly 5′ to the point of cleavage, at the major cleavage site, and directly 3′ to the point of cleavage. The dT_8 phosphorothioyl analogues or PS oligomers were purified to stereochemical homogeneity, and the position and chirality of the phosphorothiodiester linkage were verified. This study reports on the enzyme's ability to differentiate between the chiral phosphorothioyl epimers and the effects these nonbridging sulfur

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substitutions have on the distribution of products and their respective rates of formation.

Experimental Procedures

Materials

Deionized formamide was purchased from Fluka. Electrophoresis-grade TEMED, 19:1 acrylamide–*N*,*N*′-methylenebis(acrylamide), and urea were purchased from BRL. Oligodeoxynucleotide pdT_8 was purchased from Pharmacia. All reagents required for oligodeoxynucleotide synthesis were from Applied Biosystems. [γ-^{32}P]ATP (3.0 μCi/pmol) and NENSORB 20 columns were purchased from NEN. T4 polynucleotide kinase was purchased from New England BioLabs. Snake venom phosphodiesterase I from *Crotalus atrox* and alkaline phosphatase from *E. coli* were from Sigma and were used without further purification. Scinti Verse I was purchased from Fisher. All other organic reagents, buffers, and inorganic salts were commercially available reagent-grade chemicals. The sulfur (S_8) used in the synthesis of the dT_8 phosphorothioyl analogues was recrystallized from dry benzene just prior to use. Solvents were of HPLC grade (where applicable) and were degassed before and during use. In all experiments, the water was distilled and then deionized by Corning's Mega-Pure D2 system.

Methods

Purification of E. coli Topoisomerase I. *E. coli* topoisomerase I was purified from HB101 cells containing a plasmid for overexpression of the enzyme (Wang et al., 1983). The detailed procedures will be published elsewhere.

Synthesis and Purification of Oligodeoxynucleotide PS Analogues. Except as noted below, oligomers were synthesized by phosphite methodology using *O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidites (Matteucci & Caruthers, 1981; McBride et al., 1987). To substitute a nonbridging sulfur atom for an oxygen atom at a particular phosphodiester linkage during the synthesis of the PS oligomers, the automated synthetic cycle was interrupted after the tetrazole-catalyzed phosphoramidite coupling step. The phosphite was then oxidized to the *O*-(2-cyanoethyl) phosphorothiodiester at 60 °C for 15 min with a 400 mM solution of sulfur (S_8) in dry 2,6-lutidine (Stec et al., 1984). The remainder of the standard automated cycle was then completed.

The PS oligomers were purified by a two-step chromatographic procedure with a 30 × 3.9 mm μBondapak C18 column (Waters) at ambient temperature and a flow rate of 1 mL/min. The first step separated the full-length 5′-(dimethoxytrityl) product from the unsuccessful 5′-deprotected shorter products. After the 5′-(dimethoxytrityl) protecting group was removed, the second step separated the two phosphorothioyl diastereomers. Solvent A was 50 mM triethylammonium acetate buffer, pH 7.0; solvent B was acetonitrile. The gradient in step 1 was as follows: at 0 min, 20% B; at 1 min, start ramp to 35% B over 15 min. The gradient in step 2 was as follows: at 0 min, 10% B; at 1 min, start ramp to 15% B over 15 min. Then the oligomers were purified by 7 M urea–polyacrylamide gel electrophoresis and eluted from the gel.

The HPLC retention times for dT_8 and the PS oligomer epimers were determined on a 15 × 4.6 mm Zorbax C8 column (Phenomenex) at ambient temperature and at a flow rate of 1 mL/min. The solvents were the same as above. The gradient was as follows: at 0 min, 10% B; at 0 min, start ramp to 20% B over 20 min. Chromatograms were recorded with the Hewlett-Packard diode array spectrophotometer at 260 nm. Five microliters of each 35 μM oligomer was injected onto the column for analysis.

Synthesis and Purification of 5′-^{32}P-Labeled Oligodeoxynucleotide. pdT_8 and analogues were labeled at the 5′ end with ^{32}P by T_4 polynucleotide kinase. A 20-μL reaction solution containing 80 μM oligomer, 0.83 μM [γ-^{32}P]ATP (50 μCi), 5 mM DTT, 10 mM $MgCl_2$, and 10 units of T_4 polynucleotide kinase in 70 mM Tris-HCl at pH 7.5 was incubated for 30 min at 37 °C (Maniatas et al., 1978).

The 5′-^{32}P-labeled oligomer was purified from the kinase reaction mixture by selective elution from a NENSORB 20 column (from NEN). The eluant containing the purified compound was lyophilized, and the residue was dissolved in H_2O to a concentration of 25 μM (0.75 μCi/μL).

Positional Analysis of Phosphorothiodiester Linkage in Oligodeoxynucleotide PS Analogues. The position of the phosphorothiodiester linkage was verified by partial digestion with snake venom phosphodiesterase I (*C. atrox*). The 40-μL reaction solution contained 15 μM 5′-^{32}P-labeled oligomer (4.0 μCi), 2.0 mM $MgCl_2$, and 0.2 unit of phosphodiesterase I in 0.1 M Tris-HCl, pH 8.5, and the reaction was performed at ambient temperature. Aliquots of the reaction solution were quenched at various time points by mixing 5 μL of the reaction solution with 15 μL of the alkali stop solution [0.02% (w/w) bromophenol blue, 0.1 mM EDTA, 197 mM NaOH, and 79% (v/v) deionized formamide]. These quenched reaction solutions were electrophoresed as described below for the products of topoisomerase I cleavage. The polyacrylamide gel autoradiographs were scanned with a LKB Ultroscan XL laser densitometer, and the densitometry scans were analyzed by the GelScan XL laser densitometer program.

Stereochemical Analysis of Phosphorothiodiester Linkage in Oligodeoxynucleotide PS Analogues. The chirality of the phosphorothiodiester linkage was verified by complete digestion with snake venom phosphodiesterase I (*C. atrox*) and alkaline phosphatase (*E. coli*). The 50-μL reaction solution contained 50 μM oligomer, 2.0 mM $MgCl_2$, 0.1 unit of phosphatase, and 0.16 unit of phosphodiesterase I in 0.1 M Tris-HCl, pH 8.5. The reaction was incubated for 15 h at 37 °C, and then an additional 0.5 unit of phosphodiesterase I was added, and the reaction was incubated for an additional 10 h at 37 °C. The products of this enzymatic digestion were analyzed with a 15 × 4.6 mm Zorbax C8 column (Phenomenex) at ambient temperature and with a flow rate of 1 mL/min. The solvents were the same as above. The gradient was as follows: at 0 min, 1% B; at 0 min, start ramp to 25% B over 30 min. Chromatograms were recorded with the Hewlett-Packard diode array spectrophotometer at 260 nm. Ten microliters of each reaction mixture was injected onto the column.

Cleavage of 5′-^{32}P-Labeled Oligodeoxynucleotide by E. coli Topoisomerase I. The ionic strength of the cleavage reaction was maintained at 50 mM with NaCl. The 35-μL reaction solution contained 2.0 μM ^{32}P-labeled oligomer (1.6 μCi), 0 or 10 mM $MgCl_2$, 50 or 20 mM NaCl, 6 mM β-mercaptoethanol, 0.1 mg/mL gelatin, and 70 ng (20 nM) of *E. coli* topoisomerase I in 10 mM Tris-HCl, pH 7.5. A solution of all the components, minus the substrate, and the substrate solution were incubated separately for 5 min at 37 °C. The cleavage reaction, performed at 37 °C, was initiated by the addition of the substrate. Aliquots of 5 μL of the reaction solution were removed at 5-min intervals and mixed with 15 μL of the alkali stop solution. The quenched samples were then frozen. All experiments were run in triplicate.

Electrophoretic Analysis of 5′-^{32}P-Labeled Oligodeoxynucleotide Cleavage Products. Urea sequencing gels (7 M urea, 20% polyacrylamide, 50 cm × 38 cm × 0.4 mm) were preelectrophoresed for 30 min with Tris–borate buffer (Maxam

Table I: HPLC Analysis of dT_8 and the Oligodeoxynucleotide PS Analogues[a]

species	retention time (min)	% CH_3CN for elution
dT_8	6.7	13.4
(R_P)-$dT_{3(PS)}T_5$	7.9	14.0
(S_P)-$dT_{3(PS)}T_5$	8.3	14.1
(R_P)-$dT_{4(PS)}T_4$	7.8	13.9
(S_P)-$dT_{4(PS)}T_4$	8.3	14.1
(R_P)-$dT_{5(PS)}T_3$	7.8	13.9
(S_P)-$dT_{5(PS)}T_3$	8.3	14.1

[a] The purity and retention time for each oligomer were analyzed with a Zorbax C8 column at ambient temperature and at a flow rate of 1 mL/min. Solvent A was 50 mM triethylammonium acetate buffer, pH 7.0; solvent B was acetonitrile. The gradient was as follows: at 0 min, 10% B; at 0 min, start ramp to 20% B over 20 min. Chromatograms were recorded at 260 nm. The absolute configurations at the phosphorothioyl centers are given in parentheses and were asigned by sensitivity to snake venom phsophodiesterase I cleavage. See text for further details.

& Gilbert, 1980) at a constant voltage of 1500 V. Eight microliters from each quenched samples was added to each well and electrophoresed at a constant voltage of 1500 V for 2–3 h. Under these conditions, substrate and products migrate through three-fourths of the gel. The gel was transferred to Whatman 3MM paper and autoradiographed.

From the developed X-ray film, the desired bands were identified, excised, and placed in scintillation vials along with 5 mL of Scinti Verse I. The data were analyzed by least squares with RS1 from BBN Software Products Corp.

Results

The automated synthesis of oligomers is well suited for generating a host of nonbridging phosphodiester linkage derivatives. Here we have substituted the nonbridging oxygen with sulfur by oxidizing the phosphite with S_8 in 2,6-lutidine instead of with I_2 in water. The nomenclature used in this paper is as follows. In the compound (R_P)-$dT_{3(PS)}T_5$, for example, the chirality of the phosphorothioyl linkage is in parentheses, the first numerical subscript denotes the number of nucleotides 5′ to the PS linkage, and the second numerical subscript denotes the number of nucleotides 3′ to the PS linkage. With one substitution per molecule, sulfur substitutions were performed after the third, fourth, and fifth nucleotides and were named (R_P)- and (S_P)-$dT_{3(PS)}T_5$, -$dT_{4(PS)}T_4$, and -$dT_{5(PS)}T_3$, respectively.

The phosphorothioyl epimers were readily separated by HPLC reverse-phase chromatography. In all cases, the other epimer was not detected in the HPLC chromatogram monitored at 260 nm after separation. For most of the PS oligomers with data published to data, the R_P diastereomer elutes from the column before the S_P diastereomer (Domanico, 1986; Connolly et al., 1984; Stec et al., 1984). Table I shows the retention times and the calculated percentage of acetonitrile for elution from the Zorbax C8 reverse-phase column for dT_8 and all the PS oligomer epimers. Both epimers of all the PS oligomers eluted after dT_8.

The chirality of each PS oligomer was preliminary assigned by its HPLC profile. The stereochemistry as well as the position of the phosphorothiodiester linkage was then confirmed by sensitivity to snake venom phosphodiesterase I. Snake venom phosphodiesterase I, a 5′-exonuclease, cleaves oligomers to 5′-mononucleotides and prefers to cleave oligonucleotides in the 3′ → 5′ direction but does possesses endonuclease activity. This phosphodiesterase has been shown to cleave only the R_P epimer of dinucleotide phosphorothioyl analogues but at a rate 20 times slower than that of phosphodiesters (Bryant & Benkovic, 1979; Burgers et al., 1979).

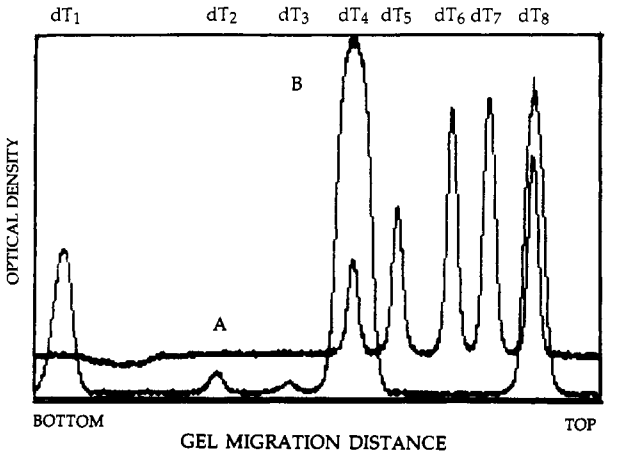

FIGURE 1: Products from the phosphodiesterase I partial digestion of (R_P)-[5′-^{32}P]$pdT_{3(PS)}T_5$: laser densitometry analysis of polyacrylamide gel. 5′-^{32}P-Labeled (R_P)-$pdT_{3(PS)}T_5$ was cleaved with snake venom phosphodiesterase I, and aliquots were quenched at various time points. The quenched reaction mixture was electrophoresed through a 7 M urea–polyacrylamide gel. The developed X-ray film was analyzed by laser densitometry at 633 nm. In curve A, the reaction solution contained 3×10^{-4} unit of phosphodiesterase I and was quenched at 45 min. In curve B, the reaction solution contained 0.15 unit of phosphodiesterase I and was quenched at 20 s. The gel was scanned down from the well, and the densitometry scan is read from right to left. The identity of each peak is indicated at the top of the graph. See text for further details.

The oligomer synthetic scheme all but excludes the possibility that sulfur substitution would occur at places other than desired. The position of the substitution was verified by partially degrading the 5′-^{32}P-labeled PS oligomers with phosphodiesterase I, the reaction products were electrophresed, and the developed X-ray film taken from the gel was analyzed by laser densitometry.

Figure 1 shows the product distribution from the partial enzymatic digestion of (R_P)-$dT_{3(PS)}T_5$ at both low (curve A) and high (curve B) enzyme concentration. Curve A shows that the products [5′-^{32}P]dT_7, -dT_5, and -dT_4 were formed in relatively equal amounts. None of the shorter products were seen at this low enzyme concentration because the enzyme cleaves R_P phosphorothiodiesters very slowly and is a poor endonuclease. Curve B shows that at a higher enzyme concentration the enzyme cleaves the PS oligomer quickly to the position of the phosphothiodiester giving [5′-^{32}P]dT_4. Then by virtue of the endonuclease activity of phosphodiesterase I, the substrate was slowly cleaved 5′ to the phosphorothiodiester linkage giving both [5′-^{32}P]dT_2 and -dT_1 products. A small amount of the [5′-^{32}P]dT_3 can be seen because the enzymes is able to cleave the R_P phosphorothiodiester. With (S_P)-$dT_{3(PS)}T_5$, the [5′-^{32}P]dT_3 product was not seen.

The stereochemistry of the PS oligomers was verified by analysis of phosphodiesterase I cleavage sensitivity. Both the incubation time and the concentration of phosphodiesterase I were increased to the point where all the phosphodiester bonds as well as the R_P phosphorothiodiester linkages were cleaved. The products were simultaneously 5′-dephosphorylated with alkaline phosphatase. Both epimers were subjected to enzymatic digestion for 15 h, and then, more phosphodiesterase I was added with an additional incubation of 10 h. A 10-μL aliquot of each reaction mixture was analyzed by reverse-phase HPLC at both the 15- and 25-h time points.

After cleavage of all the phosphodiester bonds but prior to cleavage of any phosphorothiodiester bonds, the percent area attributable to the phosphorothiodiester, on a nucleotide basis, relative to the total number of nucleotides should be 25%. For the S_P epimer, the percent area of the phosphorothiodiester

Table II: Phosphodiesterase I Digestion of (R_P)- and (S_P)-$dT_{3(PS)}T_5$: HPLC Analysis[a]

comments	species	time (min)	% area
standards	dT_1	5.4	
	dT_2	11.8	
	(R_P)-$dT_{3(PS)}T_5$	17.1	
	(S_P)-$dT_{3(PS)}T_5$	17.5	
(R_P)-$dT_{3(PS)}T_5$	dT_1	5.46	90.10
15-h digest	(R_P)-$dT_{(PS)}T$	14.26	9.90
25-h digest	dT_1	5.40	96.35
	(R_P)-$dT_{(PS)}T$	14.14	3.65
(S_P)-$dT_{3(PS)}T_5$	dT_1	5.29	76.96
15-h digest	(S_P)-$dT_{(PS)}T$	15.01	23.04
25-h digest	dT_1	5.40	77.36
	(S_P)-$dT_{(PS)}T$	14.93	22.64

[a] The (R_P)- and (S_P)-$dT_{3(PS)}T_5$ oligomers were digested simultaneously with alkaline phosphatase and phosphodiesterase I. Standard samples and the product of digestion were analyzed with a Zorbax C8 column at ambient temperature and at a flow rate of 1 mL/min. Solvent A was 50 mM triethylammonium acetate buffer, pH 7.0; solvent B was acetonitrile. The gradient was as follows: at 0 min, 1% B; at 0 min, start ramp to 25% B over 30 min Spectra were recorded at 260 nm. The absolute configurations at the phosphorothioyl centers are given in parentheses. See text for further details.

will remain at 25%, but for the R_P epimer this value should drop to 0%.

Table II presents the results of this analysis for the (R_P)- and (S_P)-$dT_{3(PS)}T_5$ species. dT_1, dT_2, and (R_P)- and (S_P)-$dT_{3(PS)}T_5$ were run as markers to identify the products of the enzymatic cleavage. dT_2 should have a similar retention time to $dT_{(PS)}T$. At the 15-h time point, a new peak was seen for each epimer. These peaks were assigned out of default as the (R_P)- and (S_P)-$dT_{(PS)}T$ species. The percent area of the (R_P)-$dT_{(PS)}T$ peak is much lower than 25%, indicating that this $dT_{(PS)}T$ species was hydrolyzed by the enzyme. As was the case when the retention times of dT_8 were compared to those of the PS analogues, both $dT_{(PS)}T$ species eluted later than dT_2. This assignment will be discussed in more detail below.

Upon further incubation of both epimers, the amount of the R_P phosphorothiodiester remaining decreased to near zero but the S_P phosphorothiodiester remained fairly constant. After an additional 5 h of incubation, the R_P phosphorothiodiester peak was no longer visible in the HPLC chromatogram. Therefore, our initial stereochemical identification by HPLC retention times was correct.

As predicted above, the percent area attributed to the S_P phosphorothiodiester was approximately 25%. A higher value would have indicated incomplete cleavage by phosphodiesterase I of the phosphodiester bonds 5′ to the phosphorothiodiester linkage. This observation then unambiguously identifies the species eluting between 14.1 and 15.0 min as the $dT_{(PS)}T$ species.

The 5′-^{32}P-labeled products from the enzymatic cleavage of dT8 and PS analogues by *E. coli* topoisomerase I were identified on urea sequencing gels by coelectrophoresis with known standards. [5′-^{32}P]dT_8 is cleaved to [5′-^{32}P]dT_4 and, to a much lesser extent, to [5′-^{32}P]dT_3 and [5′-^{32}P]dT_5. The products formed and the site of cleavage are as follows: [5′-^{32}P]dT_3, between the 3rd and 4th nucleotide; [5′-^{32}P]dT_4, between the 4th and 5th nucleotide; and [5′-^{32}P]dT_5, between the 5th and 6th nucleotide.

For dT_8 and the PS oligomers, the fraction of each product formed at each time point was determined by dividing the radioactivity from that product's gel band by the sum of the radioactivities of all the product gel bands and the substrate gel band. The rate of cleavage was determined by plotting the fraction of product formed as a function of time and multiplying the least-squares slope by the initial substrate concentration. For all data reported here the correlation value r was greater than 0.95. The product distribution was determined by dividing a product's rate of formation by the substrate's rate of cleavage.

Table III shows the rate of formation for each product and, from these rates, the percent of each product formed in the presence and absence of 10 mM Mg(II) ions. The following summary pertains to columns where Mg(II) was present in the reaction mixture. With dT_8, 82% of the substrate was cleaved to [5′-^{32}P]dT_4, 7% of the substrate was cleaved to [5′-^{32}P]dT_5, and 12% of the substrate was cleaved to [5′-^{32}P]dT_3. dT_8 was the only molecule studied that showed any significant cleavage to [5′-^{32}P]dT_5. For five of the six PS oligomers, the substrate was cleaved to one or essentially one product (>97%). With labeled (R_P)- and (S_P)-$dT_{3(PS)}T_5$, (R_P)-$dT_{4(PS)}T_4$, and (S_P)-$dT_{5(PS)}T_3$, the substrate was cleaved to [5′-^{32}P]dT_4. Enzymatic cleavage of labeled (S_P)-$dT_{4(PS)}T_4$ also yielded essentially one product, [5′-^{32}P]dT_3. Unexpectedly, (R_P)- and (S_P)-$dT_{4(PS)}T_4$ were very slowly cleaved at the phosphorothiodiester bond to yield dT_4. With (R_P)-$dT_{4(PS)}T_4$, Mg(II) ions enhanced, by a factor of 5, the rate of phospho-

Table III: Product Distribution from the Cleavage of dT_8 and dT_8 Oligodeoxynucleotide PS Analogues by *E. coli* Topoisomerase I[a]

	with 10 mM $MgCl_2$			without $MgCl_2$		
species	dT_5	dT_4	dT_3	dT_5	dT_4	dT_3
rate (×10^3)						
dT_8	0.294	3.59	0.506	0.105	1.30	0.518
(R_P)-$dT_{3(PS)}T_5$	0	5.20	0	0	5.10	0
(S_P)-$dT_{3(PS)}T_5$	0	1.84	0	0	1.16	0
(R_P)-$dT_{4(PS)}T_4$	0	0.148	0	0	0.0280	0
(S_P)-$dT_{4(PS)}T_4$	0	0.0309	8.35	0	0.0393	12.6
(R_P)-$dT_{5(PS)}T_3$	0	0.353	0.697	0	0.244	1.18
(S_P)-$dT_{5(PS)}T_3$	0	10.9	0.173	0	15.8	0.395
percentage						
dT_8	6.7	81.8	11.5	5.5	67.4	26.4
(R_P)-$dT_{3(PS)}T_5$	0	100	0	0	100	0
(S_P)-$dT_{3(PS)}T_5$	0	100	0	0	100	0
(R_P)-$dT_{4(PS)}T_4$	0	100	0	0	100	0
(S_P)-$dT_{4(PS)}T_4$	0	0.4	99.6	0	0.3	99.7
(R_P)-$dT_{5(PS)}T_3$	0	33.6	66.4	0	17.2	82.8
(S_P)-$dT_{5(PS)}T_3$	0	98.4	1.6	0	97.6	2.4

[a] The product distribution is tabulated both in absolute rates and as a percentage. The rate units are micromolar 5′-^{32}P-labeled product formed per minute. The percentage was determined from the rate of each product's formation relative to the rate of dT_8 substrate cleavage (see Table IV). The reactions were run at 37 °C with 2 μM substrate and 0.02 μM enzyme at a constant ionic strength of 50 mM. See text for further details. The products indicated are the 5′-^{32}P-labeled species.

Table IV: Rate of Cleavage of dT_8 and dT_8 Oligodeoxynucleotide PS Analogues in the Presence and Absence of 10 mM $MgCl_2$ by *E. coli* Topoisomerase I

species	rate ($\times 10^3$)[a]		relative rate[b]	
	with[c]	without	with	without
dT_8	4.39	1.93	1	1
(R_P)-$dT_{3(PS)}T_5$	5.20	5.10	1.18	2.65
(S_P)-$dT_{3(PS)}T_5$	1.84	1.16	0.42	0.60
(R_P)-$dT_{4(PS)}T_4$	0.15	0.03	0.03	0.02
(S_P)-$dT_{4(PS)}T_4$	8.38	12.7	1.91	6.58
(R_P)-$dT_{5(PS)}T_3$	1.05	1.42	0.24	0.74
(S_P)-$dT_{5(PS)}T_3$	11.1	16.2	2.53	8.43

[a] The units are micromolar [5′-^{32}P]pdT_8 hydrolyzed per minute. [b] The relative rates for the analogues are relative to the rate of dT_8 substrate cleavage. [c] With and without indicate the presence or absence of 10 mM $MgCl_2$. The reactions were run at 37 °C with 2 μM substrate and 0.02 μM enzyme at a constant ionic strength of 50 mM.

rothiodiester bond cleavage, but with (S_P)-$dT_{4(PS)}T_4$, Mg(II) ions retarded, by a factor of 1.5, the rate of phosphorothiodiester bond cleavage. Unlike the other PS oligomers, labeled (R_P)-$dT_{5(PS)}T_3$ showed two major cleavage products [5′-^{32}P]dT_4 and [5′-^{32}P]dT_3 in a 1 to 2 ratio.

The effect of Mg(II) addition on product distribution was quite complex but does show a pattern. Regardless of substrate, the rate of [5′-^{32}P]dT_4 formation was either enhanced to a greater extent or retarded to a lesser extent relative to the rate of formation of other products upon addition of Mg(II) ions.

The rate of cleavage of dT_8 and phosphorothioyl analogues to products in the presence and absence of Mg(II) ions is shown in the first column pair in Table IV. The addition of Mg(II) ions enhanced the rate of cleavage for (R_P)- and (S_P)-$dT_{3(PS)}T_5$ and (R_P)-$dT_{4(PS)}T_4$ and retarded the rate of cleavage for (S_P)-$dT_{4(PS)}T_4$ and (R_P)- and (S_P)-$dT_{5(PS)}T_3$. Two generalities can be drawn from Table IV by neglecting that (R_P)-$dT_{5(PS)}T_3$ was cleaved to two major products and (R_P)-$dT_{4(PS)}T_4$ was cleaved at the phosphorothiodiester linkage. First, the R_P epimer was preferred substrate when the phosphorothioyl linkage was 5′ to the cleavage site as in $dT_{3(PS)}T_5$, and the S_P epimer was preferred substrate when the phosphorothioyl linkage was 3′ to the cleavage site as in $dT_{5(PS)}T_3$. Second, when the phosphorothioyl linkage was 5′ to or at the cleavage site, Mg(II) ions accelerated the rate of cleavage, and when the phosphorothioyl linkage was 3′ to the cleavage site, Mg(II) ions retarded the rate of cleavage.

The difference in rates of cleavage between the R_P and S_P epimers was much greater for $dT_{4(PS)}T_4$ and $dT_{5(PS)}T_3$ than for $dT_{3(PS)}T_5$. However, this difference in rates between epimers decreased upon the addition of Mg(II) ions. Except for (R_P)-$dT_{4(PS)}T_4$, the addition of Mg(II) ions increased or decreased the rate of cleavage of all substrates by less than a factor of 2.

The second column pair relates the rate of cleavage of each PS oligomer to the rate of cleavage of dT_8. For all the PS oligomers regardless of the Mg(II) ion situation, the more reactive epimer was cleaved faster than dT_8.

Discussion

We are interested in establishing the mechanism of *E. coli* DNA topoisomerase I. An early goal is the characterization of the extent of interaction between the DNA substrate and the enzyme domain where covalent cleavage of phosphodiester backbone bond occurs.

The substrate dT_8 has been used because it is enzymatically cleaved to only a few products making kinetic analysis simple. The cleavage reactions were performed on partially 5′-^{32}P-phosphorylated substrate. We have found that cleavage kinetics is not affected by the extent to which the 5′ end of the substrate was phosphorylated (data not shown). Therefore, the partially 5′-^{32}P-labeled substrate may be considered homogeneous.

Nonbridging sulfur substituted analogues were studied for several reasons. *E. coli* topoisomerase I shows little or no sequence specificity. Therefore, the majority of protein–DNA interactions probably occur between the enzyme's active-site amino acids and the negatively charged and, most likely metal-complexed, phosphodiester backbone. Nonbridging sulfur substitution into the phosphodiester backbone uniquely alters some of the molecular properties while mimicking quite well other important aspects of the molecule. Some of the similarities and differences are as follows: (a) Sulfur substitution generates chiral phosphodiester bonds. The stereochemical sensitivity of the various enzyme binding sites and the stereochemical course of the half-reactions involved in the hydrolysis of oligomer when cleavage occurs at the phosphorothiodiester bond can then be analyzed. (b) Divalent metal ion coordination to the phosphate's ligands is metal ion dependent. For example, Mg(II) ions predominantly coordinate to the phosphate's oxygens (Jaffe & Cohn, 1978a), Cd(II) ions predominantly coordinate to the phosphate's sulfur, and Co(II) ions coordinate to either ligand (Jaffe & Cohn, 1978b). By altering the metal ion, we had anticipated the enzyme's DNA–metal isomeric preference to change. However, analysis of stereochemical differentiation based on metal complex isomers was foiled by the complete inhibition of cleavage by Co(II) and Cd(II) ions with oligomer substrates (Domanico and Tse-Dinh, unpublished results). (c) The P–S bond order is closer to 1 than the P–O bond order, thus redistributing the phosphodiester monoanion's negative charge (Frey & Sammons, 1985; Liang & Allen, 1987). In the phosphorothiodiester monoanion, the charge on sulfur is greater than the charge on oxygen. This may allow us to determine the enzyme's sensitivity to small changes in the protein–DNA electrostatic interaction. (d) Several investigators have stated that sulfur substitution causes small if any perturbations in the DNA conformation (Eckstein, 1975; Connolly et al., 1984; Frey & Sammons, 1985; Cruse et al., 1986). Therefore, the PS oligomers probably have a solution conformation that is quite similar to dT_8.

Unlike the relaxation reaction, the cleavage reaction does not require a divalent metal ion for catalysis. Although this does not mean that the metal cannot catalyze events at the covalent binding site, it does imply that the metal is required in steps other than the cleavage step. Therefore, it is interesting to note the effects that the addition of Mg(II) ions, in particular, have on the cleavage of short oligomrs.

For all substrates which can be cleaved to dT_3, the addition of Mg(II) ions decreases this product's rate of formation. With the PS oligomers, the effect of Mg(II) ions on the cleavage rate depends on whether the phosphorothiodiester bond was 5′ (increase) or 3′ (decrease) to the cleavage site. Upon comparison of the relative rates of cleavage for the epimers of a particular PS oligomer, the enzyme showed greater sensitivity to the substrates stereochemistry when the phosphorothiodiester bond was 3′ to the cleavage site, and the sensitivity was lessened by the addition of Mg(II) ions.

By reviewing the cleavage products from each PS oligomer and the rate at which one epimer was cleaved relative to the other, a hypothetical picture emerges of the enzyme's binding pocket. This hypothesis assumes that the enzyme interacts with the substrate through phosphodiester binding sites. Figure 2 is a diagram showing the proposed enzyme binding sites, P,

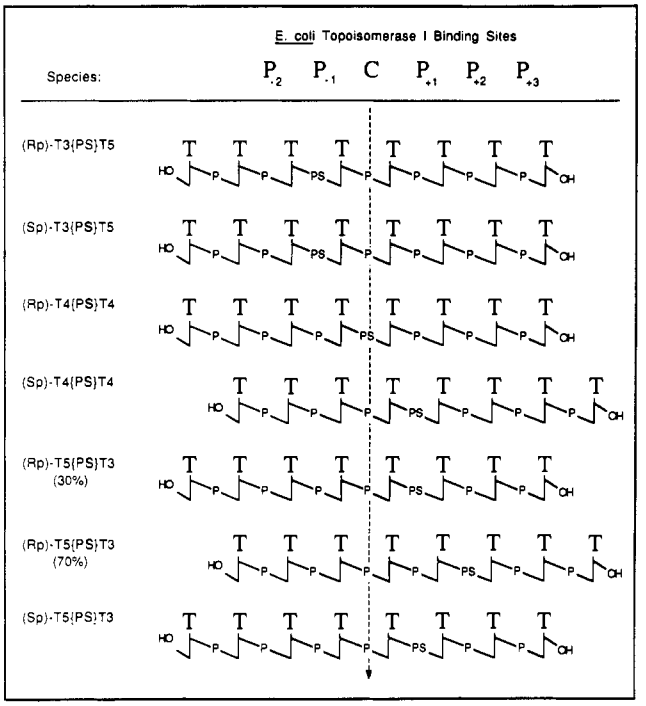

FIGURE 2: Hypothetical binding site for interactions between *E. coli* DNA topoisomerase I and the oligodeoxynucleotide PS analogues. The proposed enzyme binding sites, P_{+1}, P_{+2}, and P_{+3} and P_{-1} and P_{-2}, and the enzyme's cleavage site, C, are drawn along the top of the diagram. The P sites as drawn bind to the substrate's phosphodiester bonds. The thymidylic acid octamers are drawn as linear stick figures with the vertical lines representing the sugar moiety, the lower diagonal lines representing the 5′ linkages, and the upper diagonal lines representing the 3′ linkages. Each of the PS oligomers has been aligned beneath the enzyme's various sites so that the enzyme's cleavage site is positioned to cleave the substrate to the major observed product. Because (R_P)-$dT_{5(PS)}T_3$ was cleaved to two major products, the two binding positions for this substrate have been drawn.

and cleavage site, C, on the PS substrate analogues studied here. For each of the PS substrate analogues, the phosphodiester linkage that is cleaved by topoisomerase I has been aligned with each other. (R_P)- and (S_P)-$dT_{3(PS)}T_5$ were cleaved, but (R_P)- and (S_P)-$dT_{4(PS)}T_4$ were not cleaved 3′ to their phosphothiodiester bond. There are always at least three phosphodiester linkages 3′ to the cleavage site C. This suggests that there are three enzyme binding sites 3′ to the cleavage site, P_{+1}, P_{+2}, and P_{+3}. (S_P)-$dT_{4(PS)}T_4$ and 70% of (R_P)-$dT_{5(PS)}T_3$ were cleaved to dT_3. They have the minimal number of at least two phosphodiester linkages 5′ to the cleavage site C suggesting that there are two enzyme binding sites 5′ to the cleavage site, P_{-1} and P_{-2}. Interaction of these binding sites with the phosphodiester linkages is essential for substrate cleavage to occur. This model is consistent with the result reported previously (Tse-Dinh et al., 1983) that dA_7 was the shortest oligonucleotide tested that can be cleaved efficiently by the enzyme and the cleavage site is at the third phosphodiester linkage leaving two phosphodiester linkages 5′ and three phosphodiester linkages 3′ to the cleavage site.

Since both epimers of $dT_{3(PS)}T_5$ were cleaved to dT_4, the P_{-1} binding site is not stereospecific. (R_P)-$dT_{5(PS)}T_3$ was cleaved to dT_3 as well as dT_4, but (S_P)-$dT_{5(PS)}T_3$ was cleaved only to dT_4. This suggests that the P_{+2} site is stereospecific for the R_P isomer.

With (S_P)-$dT_{4(PS)}T_4$, the enzyme shifted its major cleavage site to yield dT_3. This shift did not occur with (R_P)-$dT_{4(PS)}T_4$, suggesting that it is unfavorable to have the R_P phosphorothiodiester at the P_{+1} site. The ratio of the rates of cleavages of (S_P)-$dT_{5(PS)}T_3$ and (R_P)-$dT_{5(PS)}T_3$ to the dT_4 product suggests that the P_{+1} binding site prefers the S_P epimer to the R_P epimer by a 31 to 1 ratio. The fact that 30% of the (R_P)-$dT_{5(PS)}T_3$ is cleaved to dT_4 suggests that other parameters, such as interaction of a P_{-3} site with the additional phosphodiester linkage when present, may also be involved in determining the sites of cleavages even though interaction with a P_{-3} site is not absolutely required for cleavage to occur.

Molecules containing phosphorothiodiester linkages usually bind poorly and are also cleaved poorly by most phosphodiesterases. Therefore, it was surprising that the more reactive of each epimer was cleaved at a rate greater than the rate of dT_8 cleavage and that both epimers of $dT_{4(PS)}T_4$ were cleaved to some extent at the phosphorothiodiester position. The rate observed was above the uncatalyzed rate of substrate hydrolysis and will allow us to determine the stereochemical course of the hydrolysis step (the cleavage step probably proceeds with inversion of configuration at phosphorus).

By using short oligomers, we hope to get a better view of catalysis at the covalent binding domain. There are caveats to this approach however. For example, the mechanistic courses taken by the relaxation and the oligomer cleavage reactions deviate. In the relaxation reaction, the 3′-OH group of the DNA backbone which is 5′ of the point of transesterification is the final nucleophile in the religation step. But that part of the oligomer which is 5′ of the point of transesterification dissociates from the enzyme (Tse-Dinh, 1986), and water is the final nucleophile in the hydrolysis step of the cleavage reaction. Another unknown is the covalent binding domain's response to catalyzing cleavage when the noncovalent binding domain is vacant, as is probably the case with short oligomers.

We have been unable to obtain accurate binding constants because of the interference of the cleavage reaction during binding measurements and the short length of the substrates. The cleavage rate to substrate concentration relationship is quite complex and will be published elsewhere. However, unpublished data from our laboratory suggest that the K_M for these oligomers is much greater than the concentration of substrate used, and, therefore, the rates reported in Table IV are proportional to k_{cat}/K_M. Future studies are needed with longer oligomers to determine the stereospecificity of the P_{-2} and P_{+3} binding sites. We are currently studying the kinetics of cleavage between *E. coli* topoisomerase I and other oligodeoxynucleotide analogues such as the phosphorotriester and phosphonotriester series as well as multiply substituted PS oligomers.

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Transcription of Left-Handed Z-DNA Templates: Increased Rate of Single-Step Addition Reactions Catalyzed by Wheat Germ RNA Polymerase II†

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ABSTRACT: Wheat germ RNA polymerase II is able to transcribe polynucleotide templates in the poly[d(G-C)] family, adopting either the right-handed B or left-handed Z conformations depending on the ionic environment and temperature. Thus, with poly[d(G-C)] either the B state (in $MgCl_2$) or the associated Z* state (in $MnCl_2$) can be established. Poly[d(G-m^{5}C)] adopts the Z form readily in $MgCl_2$, and poly[d(G-br^{5}C)] can be regarded as being "constitutively" in the Z state. In transcription studies with CpG as a primer and templates in the left-handed conformation, it is found that the rate of productive elongation, i.e., the synthesis of poly[r(G-C)], is depressed, in accordance with the results of previous studies. However, with a single triphosphate substrate, CTP, the rate of formation of the first phosphodiester bond, i.e., the synthesis of CpGpC, is about 4-fold greater with both the Z and Z* templates than with B-DNA. This transcriptional activity is also catalytic in the sense that product concentrations exceed that of the enzyme. The synthesis of CpGpC is reduced in the presence of GTP. However, the apparent K_m value for GTP utilization is lower for the trinucleotide synthesis (0.1 μM) than that obtained for productive elongation (0.8 μM), a result that also holds for B-DNA templates. All transcription reactions are specifically inhibited by the fungal toxin α-amanitin, and, in the case of the left-handed templates, by monoclonal anti-Z-DNA antibodies. The relative probabilities of single-step addition and productive elongation imply that the major distinction between transcription of templates in the B and Z conformations involves a step following the synthesis of the first phosphodiester bond. As a result, fully competent elongation complexes do not form on the left-handed DNA.

The control of gene expression at the level of transcription may be exerted, at least in part, through alterations in DNA helical conformation or topology. As an example, in vitro transcription experiments conducted with procaryotic or eucaryotic RNA polymerases have shown that the transition between B-DNA and Z-DNA is associated with a decrease in the rate of RNA synthesis on the left-handed template (van de Sande & Jovin, 1982; Durand et al., 1983; Butzow et al., 1984; Santoro et al., 1984; Peck & Wang, 1985).

In one experimental design, alternating d(C-G)$_n$ sequences were inserted downstream to promoter sites in a circular plasmid (Peck & Wang, 1985). The results indicated that the conversion of the inserts to the left-handed Z-helical form induced by negative supercoiling introduces a strong transcriptional stop signal. The polymerase, together with its nascent transcript, stops at or near the B–Z junction. That is, a few nucleotides (about four) of the alternating d(C-G)$_n$ stretch are transcribed. Transcription resumes when the insert resumes the B conformation upon relaxation of the plasmid. Such a behavior was only encountered with alternating d(C-

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