Synthesis of d(GC) and d(CG) Octamers Containing Alternating Phosphorothioate Linkages: Effect of the Phosphorothioate Group on the B–Z Transition

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ABSTRACT: The synthesis of four oligonucleotides containing alternating phosphorothioate groups, (R_P)- and (S_P)-d[G(p(S)CpG)$_3$p(S)C] and (R_P)- and (S_P)-d[C(p(S)GpC)$_3$p(S)G], by the phosphite approach is described. Silica gel to which 2′(3′)-*O*-acetyluridine and 5′-succinyl groups were bound served as support for oligomer synthesis. The syntheses were carried out by dimer addition with presynthesized diastereomerically pure dinucleoside phosphorothioates as building blocks. The products were characterized by ^{31}P NMR, nuclease P1 digestion, and oxidation to the corresponding all-phosphate-containing oligomers. The ability of each oligomer to adopt the Z conformation under high-salt conditions was screened for by circular dichroism spectroscopy. Both (R_P)-d[G(p(S)CpG)$_3$p(S)C] and (S_P)-d[C(p(S)GpC)$_3$p(S)G] are capable of forming Z-type structures at high NaCl concentrations. In the case of (R_P)-d[G(p(S)CpG)$_3$p(S)C] where a phosphorothioate of the R_P configuration occurs 5′ to a deoxycytidine residue, the B → Z transition is potentiated in comparison to the unmodified oligomer. (S_P)-d[G(p(S)CpG)$_3$p(S)C] and (R_P)-d[C(p(S)-GpC)$_3$p(S)G] retain the B conformation even at high NaCl concentration.

The right-handed double-helical form of DNA has been known for over 30 years (Watson & Crick, 1953). However, it is only during the last few years that a left-handed form of DNA has been demonstrated. The Z form was found to occur in crystals of oligonucleotides with alternating d(C-G) sequences [for review, see Rich et al. (1984)]. Previous studies had demonstrated that poly[d(G-C)] undergoes unusual conformational changes at high salt concentration that are characterized by an inversion of the CD spectrum (Pohl & Jovin, 1972) and a splitting of the phosphate resonance in the ^{31}P NMR spectrum (Patel et al., 1979; Cohen et al., 1981). Recently, Raman spectroscopy performed on alternating d-(C-G)$_3$ crystals has demonstrated a correlation between the Z-DNA conformation observed in the crystals and the high-salt form of poly[d(G-C)] (Thamann et al., 1981). The biological significance of Z DNA remains a subject of much discussion (Rich et al., 1984). However, it has been implicated in transcriptional enhancement (Rich et al., 1983) and chromosome packaging (Jovin et al., 1983a).

In an attempt to understand the mechanism of the B–Z transition, several research groups have undertaken the preparation of synthetic polynucleotides containing structurally modified nucleotides. The results of these studies have established that methyl or halogen substitution at C-5 of cytidine (Behe & Felsenfeld, 1981; Malfoy et al., 1982; Jovin et al., 1983a), bromination of C-8 in guanosine (Möller et al., 1984), and methylation at N-7 in guanosine (Möller et al., 1981) all promote the B–Z transition. Particularly interesting were those studies performed on poly[d(pCp(S)G)][1] and poly[d(pGp-(S)C)], two polymers in which a sulfur atom is stereospecifically substituted for one of two nonbridging oxygen atoms in the phosphodiester bonds. Poly[d(pGp(S)C)] having the sulfur substitution in the d(GpC) phosphodiester bond readily undergoes the salt-induced B–Z transition at salt concentrations much lower than those required to induce the transition in poly[d(G-C)]. However, in poly[d(pCp(S)G)], having the substitution in the d(CpG) phosphodiester bond, the transition is completely blocked (Jovin et al., 1983a,b; Zarling et al., 1984). As these polymers were prepared by copolymerizing dGTP and (S_P)-dCTPαS and dCTP and (S_P)-dGTPαS, respectively, with DNA polymerase I and as nucleotidyl transfer proceeds with inversion of configuration at phosphorus [see Eckstein (1983) for a review], the resultant polymers contained phosphorothioate groups of the R_P configuration.

We were interested in examining more closely the role of the phosphorothioate group in the B–Z transition and especially the effect that a phosphorothioate group of the S_P configuration might have on the equilibrium of the B and Z forms. In order to investigate these questions, we undertook the synthesis of the four phosphorothioate-containing octamers (R_P)- and (S_P)-d[G(p(S)CpG)$_3$p(S)C] and (R_P)- and (S_P)-d[C(p(S)GpC)$_3$p(S)G]. The use of R_P or S_P denotes that all the phosphorothioate groups in the oligomer are of that par-

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; dNTPαS, 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphate); dNTP, 2′-deoxynucleoside 5′-*O*-triphosphate; dNMP, 2′-deoxynucleoside 5′-*O*-phosphate; dNMPS, 2′-deoxynucleoside 5′-*O*-phosphorothioate; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; TEAB, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; DMAP, (dimethylamino)pyridine; DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz}, 5′-*O*-(*N*^{4}-benzoyl-2′-deoxycytidyl) 3′-*O*-[*N*^{2}-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosyl] *O*-methyl phosphorothioate; DMTrdC^{Bz}p(S,OCH$_3$)dG^{Ib}, 5′-*O*-(*N*^{2}-isobutyryl-2′-deoxyguanosyl) 3′-*O*-[*N*^{4}-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidyl] *O*-methyl phosphorothioate; (R_P)- and (S_P)-d[G(p(S)cpG)$_3$p(S)C], octamer consisting of alternating dGMP and dCMPS residues where R_P and S_P denote the configuration of the phosphorothioate group in d[Gp(S)C] [5′-*O*-(2′-deoxycytidyl) 3′-*O*-(2′-deoxyguanosyl) phosphorothioate]; (R_P)- and (S_P)-d[C(p(S)GpC)$_3$p-(S)G], octamer consisting of alternating dGMPS and dCMP residues where R_P and S_P denote the configuration of the phosphorothioate group in d[Cp(S)G] [5′-*O*-(2′-deoxyguanosyl) 3′-*O*-(2′-deoxyguanosyl) phosphorothioate]; poly[d(pCp(S)G)], polymer consisting of alternating dCMP and dGMPS residues; poly[d(pGp(S)C)], polymer consisting of alternating dGMP and dCMPS residues.

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ticular configuration. We report here the effect of the configuration of the phosphorothioate grups on the ability of these oligomers to adopt the Z conformation under high salt concentration.

Materials and Methods

Anhydrous pyridine and THF (maximum water content 0.01%) were purchased from Merck (Darmstadt, FRG) and stored over 4-Å molecular sieves. Dry acetonitrile was a Baker HPLC-grade reagent (J. T. Baker Chemicals, Deventer, Holland) and was stored over 3-Å molecular sieves. All other solvents used in the preparation of oligonucleotides were P.A. grade and when required dry were stored over molecular sieves. Nuclease P1 (*Penicillium citrum*, 300 units/mg) and alkaline phosphatase (calf intestine, grade 1, 1500 units/mg) were products of Boehringer Mannheim (Mannheim, FRG). Stock solutions of KH_2PO_4 (Merck, P.A. grade), for the preparation of HPLC buffers, were purified by passage over Chelex resin to remove UV-absorbing impurities (Karkas et al., 1981). HPLC buffers containing TEAB were prepared from triethylamine that was distilled first from *p*-toluenesulfonyl chloride and then redistilled from potassium hydroxide pellets.

Silica gel TLC was performed on 60 F_{254} plates supplied by Merck and eluted with $CHCl_3/CH_3OH$ (9:1 v/v) unless otherwise stated. Reverse-phase TLC was carried out on Nano-SIL C_{18}-100 plates from Macherey-Nagel (Düren, FRG). These plates were eluted with 100 mM TEAB containing 50% acetonitrile. Column chromatography was performed as indicated on either silica gel 60 (230–400 mesh) or silica gel 60 H, both supplied by Merck (Darmstadt, FRG).

Methoxydichlorophosphine was purchased from Ega Chemie (Steinheim, FRG) and used to synthesize methoxymorpholinochlorophosphine according to the procedure of McBride & Caruthers (1983). (Dimethoxytrityl)-N^2-isobutyryldeoxyguanosine was prepared by the method of Gait et al. (1982). (Dimethoxytrityl)-N^4-benzoyldeoxycytidine was obtained by the method of Schaller et al. (1963). These protected nucleosides were purified by flash chromatography (Still et al., 1978) on silica gel 60 (230–400 mesh) with $CHCl_3/CH_3OH$ mixtures as eluant.

Protected deoxyribonucleoside morpholinomethoxy phosphites were synthesized and purified according to the protocols of Dörper & Winnacker (1983) with the exception that CH_2Cl_2 was used in preference to $CHCl_3$ as the reaction solvent.

HPLC was carried out on two Waters Associates Model 6000 A pumps, controlled by a Model 680 automated gradient programmer. Analytical separations were performed on 5-μm ODS-Hypersil obtained from Shandon Southern (Runcorn, England), packed into a stainless steel column (250 × 4 mm). The column was eluted with one of the following solvent systems: system 1, a linear gradient of acetonitrile (0–35% in 20 min) in 100 mM TEAB, pH 8.0; system 2, a linear gradient of acetonitrile (0–15% in 20 min) in 50 mM KH_2PO_4, pH 6.0. A flow rate of 3 mL/min was used for both systems. Preparative purification of dimethoxytritylated oligonucleotides was carried out on a larger column (250 × 10 mm) packed with the same reverse-phase support. This column was eluted with a linear gradient of acetonitrile (0–50% in 25 min) in 100 mM TEAB with a flow rate of 5 mL/min.

^{31}P NMR spectra were recorded on a Bruker WP200SY spectrometer operating at 81.01 MHz with quadrature detection and ^1H broad-band decoupling. Chemical shifts are given in ppm and are positive when downfield from the external standard. Samples soluble in organic solvents were recorded in $CDCl_3$ and were referenced to 85% H_3PO_4. Aqueous samples were recorded as described in the text. CD spectra were recorded with a Jasco 500A spectropolarimeter connected to a Jasco 500 data processor under the conditions described in the legend to Figure 5, and the salt concentration was adjusted by the addition of solid NaCl. Absorption measurements were performed with a Shimadzu UV 240 spectrophotometer connected to a Shimadzu PR1 graphic printer. Melting curves were obtained as previously described (Pörschke & Jung, 1982).

Preparation of the Support. Merck Fractosil 200 was derivatized successively with (3-aminopropyl)triethoxysilane, succinic anhydride, and trimethylsilyl chloride as previously reported (Matteucci & Caruthers, 1981). The only change to this protocol was that succinylation was carried out in anhydrous pyridine containing an equimolar mixture of *N*-methylimidazole and succinic anhydride, according to the procedure of Efimov et al. (1983). The attachment of the uridine residue was carried out by suspending the succinylated silica (3.0 g) in dry pyridine (30 mL) containing 2′,3′-*O*-(methoxyethylidene)uridine (Fromageot et al., 1966) (0.9 g, 3.12 mmol), (dimethylamino)pyridine (0.3 g, 2.46 mmol), and DCC (2.4 g, 11.4 mmol). After this was shook for 48 h *p*-nitrophenol (1.8 g, 13.2 mmol) was added and the agitation continued for a further 20 h. The reaction was quenched by the addition of morpholine (1 mL) and the shaking was continued for another 2 h. This step is necessary to cap the unreacted carboxylic acid group. The silica was filtered, washed with methanol and then ether, and finally dried under vacuum. The 2′,3′-ortho ester intermediate was hydrolyzed to yield a mixture of the 2′- and 3′-acetates by treatment with 80% aqueous acetic acid at room temperature for 4 h. The support was analyzed both before and after acid hydrolysis by treating small amounts of the silica (10–15 mg) with 25% aqueous ammonia solution for 5 h at 50 °C. The cleavage products were then identified by silica gel TLC (eluant $CHCl_3/CH_3OH$, 85:15). As predicted, only 2′,3′-(methoxyethylidene)uridine was found in the ammonia solution prior to acid hydrolysis, while uridine was the only nucleoside found after the acid treatment. Quantitative analysis of the ammonia solution revealed that 170 μmol of uridine was bound per gram of silica.

Preparation of 3′-Protected Monomers. N^4-Benzoyl-3′-(methoxyacetyl)-2′-deoxycytidine and N^2-isobutyryl-3′-(methoxyacetyl)-2′-deoxyguanosine were prepared by the method of Potter et al. (1983a). The only deviation from the protocol was that the dimethoxytrityl group was removed by a 5-min treatment with a 2% solution of toluenesulfonic acid in $CHCl_3/CH_3OH$ (7:3 v/v) at 0 °C.

Preparation of Dinucleoside Phosphorothioates. (A) R_P and S_P Diastereomers of 5′-O-(N^4-Benzoyl-2′-deoxycytidyl) 3′-O-[N^2-Isobutyryl-5′-O-(dimethoxytrityl)-2′-deoxyguanosyl] O-Methyl Phosphorothioate. N^4-Benzoyl-3′-(methoxyacetyl)-2′-deoxycytidine (403 mg, 1 mmol) and tetrazole (280 mg, 4 mmol) were suspended in dry acetonitrile (5 mL) in a flask sealed with a septum. To the stirred suspension a solution of N^2-isobutyryl-5′-(dimethoxytrityl)-2′-deoxyguanosine 3′-*O*-(morpholinomethoxy phosphite) (1.0 g, 1.25 mmol) in dry acetonitrile (5 mL) was added dropwise from a syringe over a period of about 5 min. After 1 h sulfur (320 mg, 10 mmol) was added as a suspension in dry pyridine (15 mL) and the stirring continued for a further hour. The sulfur was then removed by filtration through a sintered glass frit (porosity 3) and the solvent removed by evaporation. The residue was dissolved in chloroform (50 mL) and extracted with solutions of saturated sodium bicarbonate (2 × 50 mL) and saturated

sodium chloride (2 × 50 mL). The organic phase was evaporated to dryness and the 3′-*O*-(methoxyacetyl) protecting group removed by treatment with dioxane (16 mL) and 25% aqueous ammonia solution (4 mL). The mixture of diastereoisomers was isolated by flash chromatography on a column of silica gel 60 equilibrated with chloroform/methanol (99:1 v/v) containing 0.1% pyridine. The diastereomers were eluted by gradually increasing the methanol concentration. Separation of the diastereomers was achieved by chromatography on a column (32 × 3.2 cm) of silica gel 60 H under elution conditions identical with those described above. Fractions of 10 mL were collected and analyzed by reverse-phase TLC. Fractions 94–105 (fast diastereomer) and fractions 108–128 (slow diastereomer) were pooled, evaporated, redissolved in dichloromethane, and precipitated into hexane. ^{31}P NMR spectroscopy in $CDCl_3$ revealed δ values of 68.27 and 70.05 ppm for the slow and fast diastereomers, respectively. Each diastereomer was obtained in an overall yield of between 25% and 30%.

(B) R_P and S_P Diastereomers of 5′-O-(N^2-Isobutyryl-2′-deoxyguanosyl) 3′-O-[N^4-Benzoyl-5′-O-(dimethoxytrityl)-2′-deoxycytidyl] O-Methyl Phosphorothioate. These were prepared by an analogous procedure. ^{31}P NMR spectroscopy in $CDCl_3$ revealed δ values of 69.84 and 69.11 ppm for the fast and slow diastereomers, respectively. The fast and slow isomers were obtained in yields of 40% and 20%, respectively.

Solid-Phase Oligonucleotide Synthesis. The 3′-*O*-(morpholinomethoxy phosphite) derivatives of the dinucleoside phosphorothioates were prepared and purified according to the method of Connolly et al. (1984). These compounds were shown to be about 95% pure by ^{31}P NMR in $CDCl_3$.

The silica support (100 mg equivalent to 17 μmol of uridine) was placed in a Hamilton 2.5-mL gas-tight syringe fitted with a glass frit (porosity 3) and an 18-cm stainless steel needle (Connolly et al., 1984). The phosphorothioate-containing oligomers were prepared by four successive coupling with the 3′-(morpholinomethoxy phosphite) derivatives of the phosphorothioate dimers. In all cases the reagents entered and left the syringe via the needle. The following synthesis cycle was used.

(1) Render the support anhydrous by washing with acetonitrile (10 × 2 mL). (2) Coupling by addition of approximately 90–100 μmol of 3′-*O*-(morpholinomethoxy phosphite) derivatives of the dinucleoside phosphorothioates in 0.5 mL of dry acetonitrile together with 0.5 mL of a saturated solution of tetrazole also in dry acetonitrile. Coupling times were 80 min for the first coupling and 50 min for the three subsequent couplings. (3) Wash with oxidation solvent (THF/H_2O/2,6-lutidine, 8:1:1 v/v) (3 × 2 mL). (4) Oxidize by addition of 1 mL of a 4% solution of iodine dissolved in the oxidation solvent, for 1 min. (5) Wash with oxidation solvent (3 × 2 mL). (6) Wash with dry THF (3 × 2 mL). (7) Cap the failed sequences by addition of 1 mL of a 7% w/v solution of (dimethylamino)pyridine in dry THF and 0.5 mL of an equivolume mixture of acetic anhydride and 2,6-lutidine, for 5 min. (8) Wash with THF (3 × 2 mL). (9) Wash with dichloroethane (3 × 2 mL). (10) Detritylate by addition of 2 mL of a 3% solution of trichloroacetic acid in dichloroethane for 3 min. (11) Wash with dichloroethane (3 × 2 mL). The syringe was periodically agitated during the coupling and cappin steps. After 11 steps the addition of one dimer unit is complete and the oligonucleotide chain is extended by returning to step 1 of the cycle.

After addition of the last dimer unit the cycle was terminated after completion of step 6. The support was washed with ether (3 × 2 mL) and dried briefly under vacuum. The methyl groups were removed from the phosphotriester in the syringe by the addition of a dioxane/triethylamine/thiophenol (2:1:1 v/v, 2 mL) mixture. After 1 h the solution was expelled from the syringe, and the support washed with methanol (3 × 2 mL) and ether (3 × 2 mL), briefly dried under vacuum, and poured into a 25-mL flask containing 3 mL of 25% aqueous ammonia solution. The flask was fitted with a septum cap, sealed firmly with wire, and heated at 50 °C for 15 h. This treatment simultaneously removes the base protecting groups and cleaves the oligomer from the support. The silica gel was removed by filtration and the filtrate evaporated at the water pump. Vigorous frothing of the solution can be prevented by cooling the solution and adding a little 1-butanol (about 5%) prior to evaporation. The residue was redissolved in 50 mM TEAB (5 mL), extracted with ether (4 × 5 mL), and once more concentrated to dryness. The resulting glass was redissolved in dioxane/0.125 M aqueous sodium hydroxide (1:4 v/v, 5 mL) and heated at 50 °C for 15 h. This treatment removes the 3′-uridine residue (van der Marel et al., 1982). The solution was treated with Merck I cation-exchange resin (ammonium form), filtered, and concentrated to a small volume. The oligomer was then purified and isolated as previously described (Connolly et al., 1984).

The oligomers $d(C\text{-}G)_4$ and $d(G\text{-}C)_4$ were synthesized by monomer addition using the 5′-*O*-(dimethoxytrityl) 3′-*O*-(morpholinomethoxy phosphite) derivatives of N^4-benzoyl-2′-deoxycytidine and N^2-isobutyryl-2′-deoxyguanosine. The support and synthesis cycles were the same as described above for the phosphorothioate oligomers. The only change to this protocol was that the reaction time for the second and subsequent couplings was reduced from 50 to 15 min. Deblocking and purification were achieved as described above for the phosphorothioate-containing oligomers.

Characterization of the Oligomers. Oligomers were characterized by digestion with nuclease P1 (Cosstick et al., 1984; Connolly et al., 1984) and analysis of the cleavage products by HPLC (solvent system 2). Retention times are summarized in Table I. The integrated peak areas (detection 254 nm) were corrected according to the method of Fritz et al. (1982). Phosphorothioate-containing oligonucleotides were additionally characterized by oxidation with a solution of iodine in aqueous pyridine (Connolly et al., 1984). The desulfurized products were isolated by HPLC (solvent system 2).

Table I: HPLC Retention Times[a]

	min		min
dG	4.8	dCMPS	0.8
dGMP	2.1	(R_P)-d[pCp(S)G]	5.2
dGMPS	2.0	(R_P)-d[Cp(S)G]	8.0
dC	3.1	(R_P)-d[pGp(S)C]	5.4
dCMP	1.1	(R_P)-d[Gp(S)C]	8.5

[a] Using solvent system 2.

RESULTS

Chemical Synthesis of Oligomers. The silica support required for the oligonucleotide synthesis was prepared by coupling the 5′-hydroxyl group of (methoxyethylidene)uridine to the carboxylic acid groups present on the succinylated silica (Figure 1). The ortho ester intermediate was subsequently hydrolyzed to give a support-bound uridine residue possessing either a free 2′- or 3′-hydroxyl group, which serves as an initiation point for oligonucleotide synthesis. The amount of uridine bound to the support was determined by treating the silica with 25% aqueous ammonia solution for 5 h at 50 °C. Spectroscopic analysis of the supernatent indicated that 170

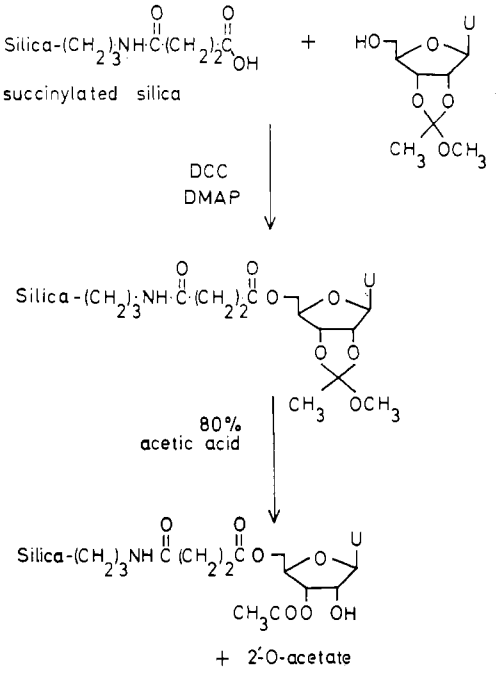

FIGURE 1: Preparation of the support.

μmol of uridine/g of silica was released. The loading capacity of the silica support (determined spectrophotometrically by the amount of dimethoxytrityl cation released after the first dimer unit had been coupled) was normally 120–130 μmol/g of support.

Octamers containing chirally pure phosphorothioate linkages were prepared by coupling presynthesized diastereomerically pure dinucleoside phosphorothioates. The required dinucleoside phosphorothioates DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz} and DMTrdC^{Bz}p(S,OCH$_3$)dG^{Ib} were prepared by condensing the appropriate deoxynucleoside 3′-(methoxymorpholino phosphite) and 3′-(methoxyacetyl)-2′-deoxynucleoside derivative.

The two diastereomers of DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz} had identical R_f values on silica gel TLC in a wide variety of solvent systems. However, fast and slow isomers were separated by careful column chromatography as described under Materials and Methods by reverse-phase TLC to analyze the fractions. ^{31}P NMR spectroscopy revealed that the fast isomer (δ = 70.05 ppm) was ≥95% pure while the slow isomer (δ = 68.27 ppm) was contaminated with 4% of the fast isomer. In the case of DMTrdC^{Bz}p(S,OCH$_3$)dG^{Gb} the fast and slow isomers were resolved with much less difficulty. ^{31}P NMR spectroscopy revealed both fast and slow isomers (δ values of 69.84 and 69.11 ppm, respectively) to be ≥98% pure.

In order to assign the absolute configuration of the dinucleoside phosphorothioates, a small amount (15 mg) of the fast and slow isomers of each dimer was deblocked (Connolly et al., 1984) and the assignment made according to the following established criteria: the S_P diastereomer is cleaved by nuclease P1 while the R_P diastereomer remains undigested (Potter et al., 1983b); ^{31}P NMR spectroscopy has revealed that the S_P diastereomer of dinucleoside phosphorothioates always resonates to higher field than the R_P diastereomer and the S_P diastereomer always has a greater retention time on reverse-phase HPLC than the R_P diastereomers (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). The results presented in Table II clearly demonstrate that for both DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz} and DMTrdC^{Bz}p(S,OCH$_3$)dG^{Ib} the fast isomer of the triester correspond to the dinucleoside phosphorothioate of the R_P configuration, and the slow to that of the S_p configuration (Figure 2).

Table II: Analytical Data on the Deblocked Phosphorothioate Dinucleosides Derived from "Fast" and "Slow" Triester Isomers

	^{31}P NMR (ppm)[a]	R_T (min)[b]	nuclease P1 digestion	configuration
d[Gp(S)C] from fast isomer of triester	55.59	11.2	not cleaved	R_P
d[Gp(S)C] from slow isomer of triester	55.13	12.7	cleaved	S_P
d[Cp(S)G] from fast isomer of triester	55.56	10.8	not cleaved	R_P
d[Cp(S)G] from slow isomer of triester	54.74	12.4	cleaved	S_P

[a] Measured in 10 mM Tris-HCl, pH 8.5, containing 1 mM EDTA, 10 mM NaCl, and 50% D_2O. Samples were referenced to 85% H_3PO_4. [b] HPLC retention times in solvent system 2.

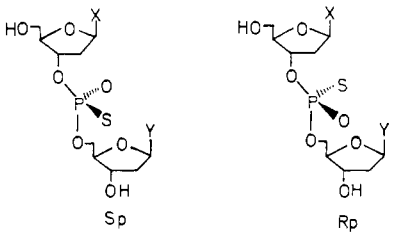

FIGURE 2: Configuration of the S_P and R_P diastereomers of the dinucleoside phosphorothioates.

The synthesis of the oligonucleotides was achieved by coupling of dimer units to the support; the coupling reactions proceeded in approximately 85% yield. After removal of the phosphate and base protecting groups, the linkage between the 2′- and 3′-hydroxyl group and the 3′-phosphate of the oligomer was readily cleaved under alkaline conditions to yield the dimethoxytritylated oligomer and uridine cyclic 2′,3′-phosphate. Starting from 100 mg of silica support between 150 and 210 A_{260} units of the oligomer were finally isolated. This corresponds to between 2.3 and 3.0 μmol of oligomer assuming an extinction coefficient of 70 600 per strand at 260 nm (Kastrup et al., 1978). The percentage yields were 18–25% on the basis of the amount of dimethoxytrityl cation released after the first coupling.

Characterization of Oligomers. The purity of the isolated oligomers was established by HPLC in solvent systems 1 and 2. In every case the oligonucleotides appeared ≥95% pure. As expected, (S_P)- and (R_P)-d[G(p(S)CpG)$_3$p(S)C] had a retention time greater than the all oxygen containing oligomer. However, no separation was observed between (S_P)- and (R_P)-d[G(p(S)CpG)$_3$p(S)C]. The situation was the same for the series of oligomers containing a 5′-deoxycytidine residue; no separation was obtained between (S_P)- and (R_P)-d[C(p(S)GpC)$_3$p(S)G], but their retention time was greater than that of the all oxygen containing oligomer. Both the all phosphate containing oligomers, d(C-G)$_4$ and d(G-C)$_4$ coeluted on reverse-phase HPLC (solvent systems 1 and 2) with the standard oligomers purchased from P-L Biochemicals.

The oligomers were further characterized by digestion with nuclease P1 followed by HPLC analysis. Nuclease P1 cleaves nucleotides to give nucleoside 5′-phosphates and also cleaves the S_P configuration of dinucleoside phosphorothioates to give 5′-phosphorothioates (Potter et al., 1983b). Thus, digestion of (S_P)-d[G(p(S)CpG)$_3$p(S)C] yields dG, dGMP, and dCMP(S) in a ratio of 1:3:4, respectively. As the starting (S_P)-dinucleoside phosphorothioate was contaminated by approximately 4% of the R_P isomer, a corresponding amount of (R_P)-d[pGp(S)C] was also detected in the digestion mixture. Digestion of (R_P)-d[G(p(S)CpG)$_3$p(S)C] yielded (R_P)-d[Gp(S)C] and (R_P)-d[pGp(S)C] in a ratio of 1:3, respectively.

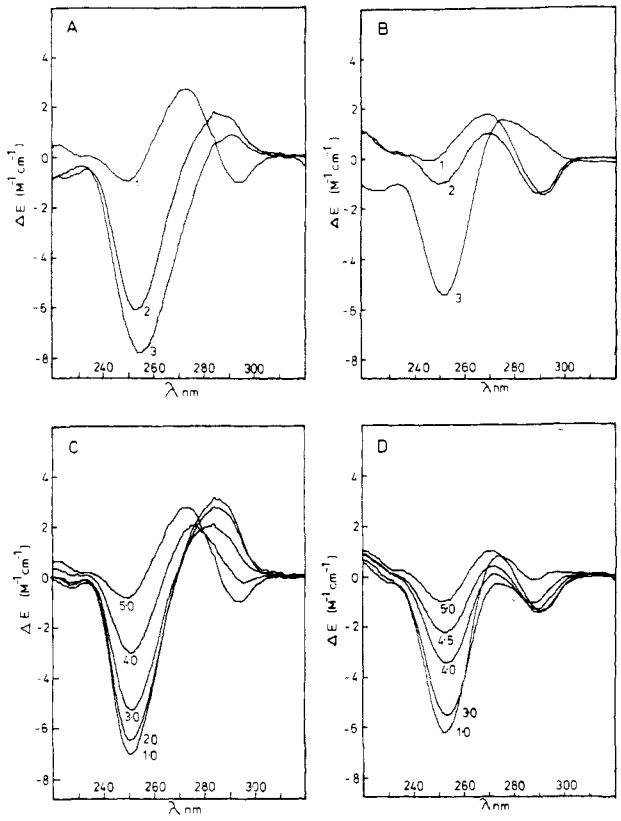

FIGURE 3: CD curves recorded in 1 mM KH_2PO_4, pH 7.0, and NaCl concentrations as stated at 20 °C. (A) (1) (R_P)-d[Gp(S)CpG)$_3$p(S)C]; (2) d(G-C)$_4$; (3) (S_P)-d[G(p(S)CpG)$_3$p(S)C] in 5 M NaCl. (B) (1) d(C-G)$_4$; (2) (S_P)-d[C(p(S)GpC)$_3$p(S)G]; (3) (R_P)-d[C(p(S)-GpC)$_3$p(S)G] in 5 M NaCl. (C) CD curves for (R_P)-d[G(p(S)-CpG)$_3$p(S)C] recorded at 1.0, 2.0, 3.0, 4.0, and 5.0 M NaCl. (D) CD curves for (S_P)-d[C(p(S)GpC)$_3$p(S)G] recorded at 1.0, 3.0, 4.0, 4.5, and 5.0 M NaCl.

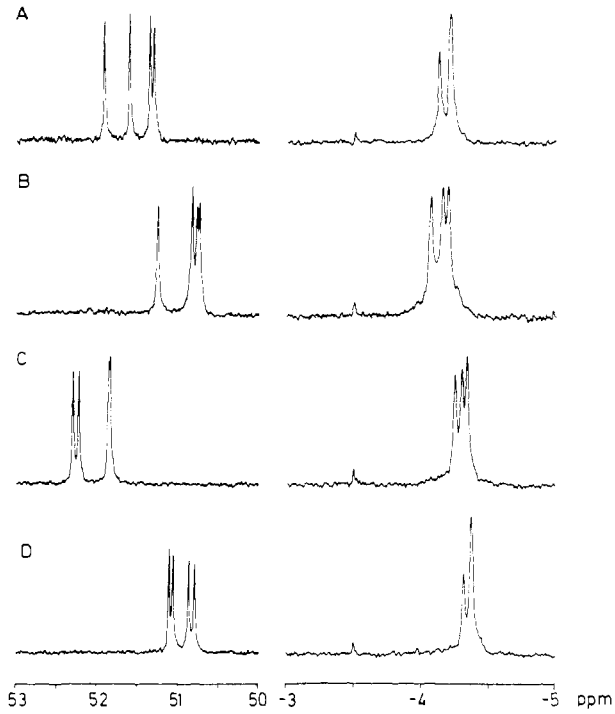

FIGURE 4: ^{31}P NMR spectra of phosphorothioate octamers. (A) (R_P)-d[G(p(S)CpG)$_3$p(S)C]; (B) (S_P)-d[G(p(S)CpG)$_3$p(S)C]; (C) (R_P)-d[C(p(S)GpC)$_3$p(S)G]; (D) (S_P)-d[C(p(S)GpC)$_3$p(S)G]. The samples contained 40 A_{260} units dissolved in 500 μL of 25 mM Hepes/NaOH, pH 8.0, containing 50 mM NaCl, 25 mM EDTA, and 50% D_2O as solvent. Samples were referenced to trimethyl phosphate.

The (R_P)-d[pGp(S)C] was unequivically identified by further treatment of the incubation mixture with alkaline phosphatase and comparison of the resultant (R_P)-d[Gp(S)C] with standard material. Similarly, the nuclease P1 digestion of (S_P)-d[C-(p(S)GpC)$_3$p(S)G] gave dC, dCMP, and dGMP(S) in a ratio of 1:3:4, respectively, and (R_P)-d[C(p(S)GpC)$_3$p(S)G] gave (R_P)-d[Cp(S)G] and (R_P)-d[pCp(S)G] in a ratio of 1:3, respectively. d(G-C)$_4$ and d(C-G)$_4$ were also digested with nuclease P1 to give the expected ratios of dC, dGMP, and dCMP.

The phosphorothioate-containing oligomers were desulfurized to give the all phosphate containing nucleotides in almost quantitative yield by treatment with iodine in aqueous pyridine (Connolly et al., 1984). Desulfurization of (S_P)- and (R_P)-d[G(p(S)CpG)$_3$p(S)C] and (S_P)- and (R_P)-d[C(p(S)-GpC)$_3$p(S)C] gave products that coeluted on HPLC (solvent systems 1 and 2) with d(G-C)$_4$ and d(C-G)$_4$, respectively.

CD Spectral Studies. The CD spectra of the oligomers were measured in 1 mM KH_2PO_4, pH 7.0, with varying concentrations of sodium chloride. Figure 3A shows the CD spectra at high salt concentration for the series of oligomers that have a 5′-deoxyguanosine residue. Both d(G-C)$_4$ and (S_P)-d[G-(p(S)CpG)$_3$p(S)C] show high-salt spectra that are almost identical with the low-salt spectra and are consistent with a right-handed helix of the B type (Pohl & Jovin, 1972; Pohl, 1976). However, with increasing salt concentrations the spectrum of (R_P)-d[G(p(S)CpG)$_3$p(S)C] shows a decrease in the intensity of the negative 253-nm band and the appearance of a new negative band at 295 nm (Figure 3C). The curve is very characteristic of the Z form (Pohl & Jovin, 1972). The midpoint of the transition, estimated by plotting the intensity of the negative band at 253 nm against NaCl concentration (not shown), occurs at approximately 3.5 M NaCl. The CD spectra for the series of oligomers that have a 5′-deoxycytidine residue are shown in Figure 3B. In 5 M NaCl both d(C-G)$_4$ and (S_P)-d[C(p(S)GpC)$_3$p(S)G] exhibited spectra characteristic of the Z form. The transition from the B to the Z form for the latter as a function of NaCl concentration is presented in Figure 3D. The midpoints of the transitions were estimated to be 3 and 4 M NaCl, respectively. The CD curve obtained for (R_P)-d[C(p(S)GpC)$_3$p(S)G] is consistent with the oligomer retaining a right-handed helical conformation even at 5 M NaCl (Figure 3B).

The ^{31}P NMR spectra of the oligomers were recorded at 10 °C as described in the legend to Figure 4. The spectra of the phosphorothioate oligomers revealed three resonances between −4 and −4.5 ppm and four resonances between 50.7 and 52.3 ppm for the phosphate and phosphorothioate groups, respectively (Figure 4). In both oligomer sequences the phosphorothioate resonances for the oligomers containing phosphorothioate groups of the S_P configuration were upfield from those of the corresponding oligomer possessing the R_P configuration. This result is consistent with observations regarding the ^{31}P NMR of the dinucleotide phosphorothioates in which the S_P isomer always resonates upfield from the R_P isomer (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). The spectra of both d(C-G)$_4$ and d(G-C)$_4$ showed a group of resonances between −3.9 and −4.2 ppm. Attempts to obtain ^{31}P NMR spectra of the oligomers in the Z conformation at high salt (3.5–4.0 M NaCl) were unsuccessful since under these conditions the oligomers were only sparingly soluble.

The thermal stability of the oligomers was determined spectrophotometrically. The results are summarized in Table III.

Table III: T_m Values for Synthesized Oligonucleotides

oligomer[a]	T_m (°C)	oligomer[a]	T_m (°C)
d(G-C)$_4$	56	d(C-G)$_4$	55
(R_P)-d[G(p(S)-CpG)$_3$p(S)C]	46	(R_P)-d[C(p(S)-GpC)$_3$p(S)G]	54
(S_P)-d[G(p(S)-CpG)$_3$p(S)C]	51	(S_P)-d[C(p(S)-GpC)$_3$p(S)G]	55

[a] Solutions contained 0.5 A_{260} OD/mL in 20 mM sodium citrate, pH 7.0, containing 20 mM NaCl.

Discussion

The interesting observation that poly[d(pGp(S)C)] in which a phosphorothioate group of the R_P configuration is at the 5′-position of deoxycytidine very readily undergoes the B–Z conformational change whereas poly[d(pCp(S)G)] where this group is at the 5′-position of deoxyguanosine is unable to adopt the Z conformation (Jovin et al., 1983a,b; Zarling et al., 1984) raises the question as to how a phosphorothioate group influences this transition. As an answer to this question may provide insight into some of the forces driving this process in general, we set out to investigate the role of the phosphorothioate group in this context more closely. As a first priority, we decided to study whether the configuration of the phosphorothioate group influences the transition. Thus, ideally we would have liked to have available phosphorothioate analogues of poly[d(G-C)] containing the phosphorothioate groups of the S_P configuration. Unfortunately, polymers containing phosphorothioate groups of the S_P configuration cannot be synthesized enzymatically as all DNA polymerases investigated so far produce phosphorothioate internucleotidic linkages of the R_P configuration in the polymer (Eckstein, 1983). It was, therefore, decided to synthesize chemically d(G-C) and d(C-G) octamers containing phosphorothioate groups of either the R_P or S_P configuration either 5′ to deoxyguanosine or 5′ to deoxycytidine. As we had been successful earlier in synthesizing an octamer containing one phosphorothioate group on a solid support by the phosphite method (Connolly et al., 1984), we decided to apply this strategy to the synthesis of these oligomers.

The most popular strategy for the synthesis of oligonucleotides on solid phase involves the derivatization of a support with the nucleoside that is destined to become the 3′-terminus of the oligomer. In order to ensure that the support has a high loading capacity, a large amount of nucleoside has to be used in the derivatization reaction (normally between 0.4 and 1.0 mmol/g of support). Clearly, this requirement is unsatisfactory for the preparation of phosphorothioate oligomers by dimer addition since a large amount of the precious dinucleoside building blocks would be wasted during the derivatization.

A strategy for solid phase synthesis of oligonucleotides that eliminates these problems has been previously reported by Gough et al. (1983a). A mixture of 2′- and 3′-*O*-benzoyluridine is anchored via its 5′-hydroxyl group to controlled pore glass, and the free 2′- or 3′-hydroxyl groups serve as the initial coupling points for oligonucleotide synthesis. A similar idea with an "inverted" uridine residue attached to a cellulose support has been exploited by Crea & Horn (1980) and van der Marel et al. (1982). In both cases a separate uridine-containing dimer had to be prepared for each type of 3′-terminus. We were interested in adapting the methodology of Gough et al. to silica supports, which have a greater capacity than controlled pore glass. In order to simplify this procedure, we chose to succinylate the silica gel by established procedures (Matteucci & Caruthers, 1981; Efimov et al., 1983) and couple the 2′,3′-*O*-(methoxyethylidene)uridine directly to the support. This procedure reduces the number of chemical conversions performed on the support to one simple step.

The oligomers were synthesized by the phosphite methodology developed by Caruthers (Matteucci & Caruthers, 1981; McBride & Caruthers, 1981). This method has been successfully employed previously for the synthesis of oligonucleotides containing one or two phosphorothioate groups (Connolly et al., 1984; Stec et al., 1984).

The phosphorothioate group can be introduced by oxidation of the support-bound intermediary nucleoside phosphite with sulfur. The mixture of diastereomers resulting from the nonspecific addition of sulfur can in many cases be separated by HPLC (Connolly et al., 1984; Stec et al., 1984). Clearly, however, this strategy is not applicable to the synthesis of oligomers containing four phosphorothioate groups, giving rise to a mixture of 16 diastereomers. In order to control the stereochemistry at the phosphorothioate groups, we adopted the approach of coupling preformed and suitably protected stereochemically pure phosphorothioate dimers (Connolly et al., 1984). On average, we obtained yields of 85% for the coupling of the phosphorothioate dimers. These yields are somewhat less than those normally obtained in the coupling of monomer units where yields of 95% or greater are commonly achieved. However, since our value of 85% for the addition of a dimer unit is equivalent to two monomer coupling steps averaging 92%, these yields are quite acceptable for the synthesis of oligomers containing up to 12 or perhaps even more residues.

Once deprotection of the phosphate and base moieties is complete, the "inverted" uridine residue can be removed from the 3′-end of the oligomer by a variety of reagents. We chose to use the alkaline hydrolysis procedure reported by van de Marel (1982). The 3′–2′ or 3′–3′ phosphodiester bonds are particularly labile at elevated pH because the neighboring hydroxyl group promotes hydrolysis through anchimeric assistance. This hydrolysis mechanism is well established from studies conducted on the alkaline degradation of RNA. It has been reported that strong alkali causes partial deamination of cytidine residues (Kochetkov & Budovski, 1972). However, the nuclease P1 digests of the purified oligomers did not reveal any extraneous peaks that could be attributed to deoxyuridine monophosphate or deoxyuridine-containing dimers. Other reported methods for the cleavage of this type of phosphodiester bond include enzymatic hydrolysis with pancreatic ribonuclease (van de Marel et al., 1982) and hydrolysis at neutral pH in the presence of divalent lead ions (Farkas, 1968; Gough et al., 1983b). We were particularly anxious to avoid the use of lead salts in case they proved difficult to remove from the phosphorothioate oligomers.

The work of Thamann et al. (1981) correlating the high-salt form of poly[d(G-C)] with the left-handed Z-DNA structure of crystalline d(C-G)$_3$ through laser Raman studies has established CD spectroscopy as a simple and reliable method for the detection of Z DNA. We therefore chose to use this technique as a means of investigating the influence of phosphorothioate substitution on the B–Z transition of d(G-C) and d(C-G)$_4$. The choice of these oligonucleotides was dictated by the desire to have the same length oligonucleotide with the same number of phosphorothioate groups either 5′ to deoxyguanosine or 5′ to deoxycytidine. A result of this strategy is that we compare an oligonucleotide, d(C-G)$_4$, that adopts the Z conformation with another, d(G-C)$_4$, that does not. Figure 3A shows the effect of phosphorothioate linkages of the R_P and S_P configuration 5′ to a deoxycytidine residues in d(G-C)$_4$. The R_P configuration (curve 1) greatly potentiates the for-

Table IV: Summary of Position and Configuration of the Phosphorothioate Group on B–Z Transition[a]

type of internucleotidic linkage	oligomer d(G-C)$_4$	oligomer d(C-G)$_4$
all phosphate	–	+
d[Gp(S)C]		
R_P	+	
S_P	–	
d[Cp(S)G]		
R_P		–
S_P		+

[a] (+) indicates B to Z conformational change under high-salt concentration; (–) indicates no change of conformation observed.

mation of Z DNA as compared to the all phosphate oligomer d(G-C)$_4$. Jovin et al. (1983a,b) have previously obtained the same result from studies conducted on poly[d(G-C)] and poly[d(pGp(S)C)]. Like d(G-C)$_4$, (S_P)-d[G(p(S)CpG)$_3$p(S)C] (Figure 3A, curves 2 and 3, respectively) retains a right-handed helix at 5 M NaCl, thus demonstrating that a phosphorothioate group of the S_P configuration at this position, unlike the R_P configuration, does not potentiate the B–Z transition. Since d(G-C)$_4$ also remains in the B form at high salt, it is impossible to assess whether the S_P configuration 5′ to deoxycytidine residues has either a more or less neutral effect or strong inhibitory effect on the transition.

The effect of phosphorothioate substitution 5′ to deoxyguanosine residues in d(C-G)$_4$ is shown in Figure 3B. A phosphorothioate group of the R_P configuration (curve 3) completely inhibits the B–Z transition: this was previously known from studies conducted on poly[d(pCp(S)G)] (Jovin et al., 1983a,b). However, the analogous oligomer containing a phosphorothioate of the S_P configuration (curve 2) is capable of adopting a left-handed helix, although there is a slight inhibitory effect as compared to unmodified d(C-G)$_4$. Thus, we have the interesting result summarized in Table IV that the configuration of the phosphorothioate groups determines whether these oligonucleotides adopt the right- or left-handed conformation.

The observation that short oligomers with the sequence d(G-C)$_n$ ($n \leq 6$) do not assume the left-handed conformation (Figure 3A, curve 2) has previously been noted (Albergo & Turner, 1981; Gorenstein et al., 1982; Reid et al., 1983; Quadrifoglio et al., 1983). In contrast, oligomers with the d(C-G)$_n$ sequence ($n \geq 2$) adopt the Z conformation at NaCl concentrations only slightly greater than those required to bring about the transition in poly[d(G-C)] (Thomas & Peticolas, 1984). The effect of the base sequence on the relative stability of the B and Z forms for the d(G-C)$_n$ and d(C-G)$_n$ series of oligomers has been the subject of two recent reports (Quadrifoglio et al., 1984; Thomas & Petricolas, 1984). Quadrifoglio et al. (1984) have shown that for the B forms the lower free energy of the d(G-C)$_n$ (n = 3 or 4) series is probably partially responsible for their unwillingness to adopt the Z conformation while Thomas & Petricolas (1984) have proposed two types of stacking interaction which possibly serve to stabilize the short d(C-G)$_n$ oligomers in the Z state. It therefore appears that enhanced stabilization of the B form and destabilization of the Z conformation both conspire to prevent the formation of the Z-type helices for the small d(G-C)$_n$ oligomers.

What are the influences that the phosphorothioate groups might exert on the B-Z conformational equilibrium? Inspection of models shows that the sulfur of phosphorothioate groups of the R_P configuration in polymers or oligonucleotides of the B conformation points into the major groove whereas in groups of the S_P configuration it is located directly on the face of the sugar–phosphate backbone. Although it is difficult to imagine that this would be the cause of differences in stability, the melting points (Table III) of the B-type conformers of the oligomers indicate that phosphorothioate groups can destabilize the B-type conformers of the oligomers. Thus, phosphorothioate groups of the R_P configuration 5′ to deoxycytidine lower the midpoint of transition of d(G-C)$_4$ by 10 °C and those of the S_P configuration by 5 °C. On the other hand, phosphorothioate groups 5′ to deoxyguanosine seem to have little or no effect on the melting of d(C-G)$_4$. It is possible that a decrease in thermal stability of the B conformer is responsible for a more facile shift of the equilibrium to the Z conformer in the case of (R_P)-d[Gp(S)CpG)$_3$p(S)C], although this is evidently not so for (S_P)-d[Cp(S)GpC)$_3$p(S)G], which does not have a decreased thermal stability but also adopts the Z conformation. It therefore appears that destabilization of the B structure alone cannot account for the behavior of these oligonucleotides, and thus, the influence that the sulfur atom has on the Z conformation also has to be considered. The X-ray structural analysis of the Z conformation of four crystal forms of d(C-G)$_3$ (Wang et al., 1981, 1984; Drew & Dickerson, 1981) reveals that d(GpC) linkages prefer the Z_I over the Z_{II} conformation. In the Z_I conformation there is a water molecule bridging guanosine N_2 and the *pro*-R_P oxygen of the d(GpC) phosphate group 3′ to the same dG residue. In the Z_{II} conformation this *pro*-R_P oxygen is hydrogen bonded to a water molecule in the octahedral coordination shell surrounding a magnesium ion, which is in turn complexed in N7 of guanine. In (R_P)-d[Gp(S)CpG)$_3$p(S)C], the *pro*-R_P oxygens in the d(GpC) linkage are replaced by sulfur. As hydrogen bonds to sulfur are normally considered to be weaker than those to oxygen, the hydrogen-bonding schemes described above would be weaker for the R_P configuration of the d[Gp(S)C] linkage. However, this diastereomer adopts the Z conformation quite readily in contrast to the S_P diastereomer and the unmodified d(G-C)$_4$, which remain in the B conformation. Thus, we have to conclude that there are other forces effecting this equilibrium. There are no such direct interactions of the *pro*-R_P or *pro*-S_P oxygens of the d(CpG) phosphate group detectable in either the Z_I or Z_{II} conformation from the X-ray structure.

Can it be envisaged that the effect of the sulfur is exerted in a more indirect manner? The relative instability of the Z conformation in comparison to the B conformation is partly caused by the closer proximity of phosphate groups in the former (Wang et al., 1979; Rich et al., 1984). Inspection of models of the B conformers of poly[d(G-C)] containing phosphorothioates of the R_P configuration 5′ to deoxycytidine and 5′ to deoxyguanosine [see foldout in Jovin et al. (1983a)] reveal that in the latter case the sulfur of the phosphorothioates in opposite strands comes closer in space than in the former. As a phosphorothioate group of the R_P configuration 5′ to deoxyguanosine prevents the adoption of the Z conformation, one is tempted to make use of this proximity to explain this result. Evidence seems to accumulate that the negative charge of a phosphorothioate is mainly localized on the sulfur (Frey & Sammons, 1985; Iyengar et al., 1985). Thus, concentration of negative charges in the Z conformer might prevent the B → Z transition. Unfortunately, this argument does not hold for (S_P)-d[(Cp(S)GpC)$_3$p(S)G], where the sulfurs in the Z conformation seem to come almost as close in space but this oligomer easily adopts the Z conformation. However, it has to be emphasized that these models were built with the coordinates of d(C-G)$_3$ (Wang et al., 1979) and that the structure

of the phosphorothioate analogues might be somewhat different. A recent publication by Soumpasis (1984) that explains the B–Z conformational change on the basis of interaction of phosphate groups and a diffuse cloud of ions has emphasized the importance of charge distribution.

A further possible explanation for our findings may result from the increased hydrophobic character of the oligomers that occurs on replacement of an oxygen atom for a sulfur atom in the phosphodiester bond. The hydration of an oligonucleotide plays an important role in the preference for a particular conformer (Rich et al., 1984). This is probably most convincingly demonstrated by the influence of 5-methylation of deoxycytidine and its preference for the Z conformation (Fujii et al., 1982; Wang et al., 1984). Unfortunately, however, this factor cannot be assessed at present for the phosphorothioate oligomers.

In summary, we are left with the unsatisfactory situation that we cannot offer an explanation for our results, which indicate such a striking influence of the configuration of the phosphorothioate group on the B–Z equilibrium. It is hoped that X-ray structural analysis of these oligomers will provide a more detailed picture of their structures and provide insight into their different behavior.

Acknowledgments

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Registry No. S, 7704-34-9; DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz} (R_P diastereomer), 96666-98-7; DMTrdG^{Ib}p(S,OCH$_3$)dC^{Bz} (S_P diastereomer), 96744-26-2; DMTrdC^{Bz}p(S,OCH$_3$)dG^{Ib} (R_P diastereomer), 96667-01-5; DMTrdC^{Bz}p(S,OCH$_3$)dG^{Ib} (S_P diastereomer), 96744-27-3; (R_P)-d[G[p(S)CpG]$_3$p(S)C], 96667-02-6; (S_P)-d[G[p(S)CpG]$_3$p-(S)C], 96744-28-4; (R_P)-d[C[p(S)GpC]$_3$p(S)G], 96667-03-7; (S_P)-d[C[p(S)GpC]$_3$p(S)G], 96744-99-9; d(G-C)$_4$, 80458-01-1; d-(C-G)$_4$, 89991-79-7; N^4-benzoyl-3′-(methoxyacetyl)-2′-deoxycytidine, 96666-99-8; N^2-isobutyryl-5′-(dimethoxytrityl)-2′-deoxyguanosine 3′-*O*-(morpholinomethoxy phosphite), 96667-00-4.

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Axial Ligands of Chloroplast Cytochrome *b*-559: Identification and Requirement for a Heme-Cross-Linked Polypeptide Structure†

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ABSTRACT: Optical, resonance Raman, and electron paramagnetic resonance spectroscopies have been used to characterize the ligands and spin state of the chloroplast cytochrome *b*-559. The protein was isolated from both maize and spinach in a low-potential form. The spectroscopic data indicate that the heme iron in both ferric and ferrous cytochrome *b*-559 is in its low-spin state and ligated in its fifth and sixth coordination positions by histidine nitrogens. Electron paramagnetic resonance data for the purified spinach cytochrome are in good agreement with those determined by Bergström and Vänngård [Bergström, J., & Vänngård, T. (1982) *Biochim. Biophys. Acta 682*, 452-456] for a low-potential membrane-bound form of cytochrome *b*-559. The *g* values of high-potential cytochrome *b*-559 are shifted from those of its low-potential forms; this shift is interpreted as arising from a deviation of the planes of the two axial histidine imidazole rings from a parallel orientation. The model is consistent with the physical data and may also account for the facility with which cytochrome *b*-559 can be converted between low- and high-potential forms. Recent biochemical and molecular biological data [Widger, W. R., Cramer, W. A., Hermodson, M., Meyer, D., & Gullifor, M. (1984) *J. Biol. Chem. 259*, 3870-3876; Herrmann, R. G., Alt, J., Schiller, D., Cramer, W. A., & Widger, W. R. (1984) *FEBS Lett. 179*, 239-244] have shown that two polypeptides, one with 83 residues and a second with 39 residues, most likely constitute the protein portion of the cytochrome. Each of the polypeptides, however, contains only a single histidine. Thus, to provide the bis(histidine) axial ligation required by the physical data, we postulate that the heme acts as a cross-linker by coordinating histidines from two different polypeptide chains.

The chloroplast cytochrome *b*-559 is a membrane protein intrinsic to the core complex of photosystem II (PSII). Although this association and other data (Butler, 1978; Matsuda & Butler, 1983) provide circumstantial links between this protein and the water-splitting, oxygen-evolving function of PSII, elucidation of its function has proven difficult (Cramer et al., 1981). A 9-10-kilodalton (kDa)[1] polypeptide associated with this cytochrome was recently purified both from maize (Metz et al., 1983) and from spinach chloroplasts (Widger et al., 1984a). The purified cytochrome was used to determine

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[1] Abbreviations: kDa, kilodalton(s); SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Chl, chlorophyll; EPR, electron paramagnetic resonance; LDS, lithium dodecyl sulfate.

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