Effect of P-Chirality of Internucleotide Bonds on B−Z Conversion of Stereodefined Self-Complementary Phosphorothioate Oligonucleotides of the [PS]-d$(CG)_4$ and [PS]-d$(GC)_4$ Series†

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ABSTRACT: Diastereomerically pure, partially modified (in selected positions) or fully modified phosphorothioate oligomers of the [PS]-d$(CG)_4$ and [PS]-d$(GC)_4$ series were investigated with respect to their ability to adopt the left-handed conformation at high sodium chloride concentration. NaCl induces the B−Z transition of [All-S_PR_P-PS]-d$(CG)_4$ with a midpoint of transition at ca. 2 *M*, which is approximately 1 M less than for unmodified d$(CG)_4$. Also, [All-R_PS_P-PS]-d$(GC)_4$ at 5 *M* NaCl converts to the Z form to the extent of ca. 55%, while the unmodified d$(GC)_4$ counterpart does not convert at all. This enhanced ability of stereodefined phosphorothioate oligomers to adopt the Z conformation is discussed in terms of already known structural factors (hydrogen bonding and water bridges) facilitating the B−Z transition, identified for unmodified d$(CG)_n$ oligonucleotides. By CD spectroscopy, the [All-S_P-PS]-d$(CG)_4$ oligomer at a NaCl concentration higher than 0.01 M adopts a unique conformation as assessed from the presence of an additional negative band centered at 282 nm.

DNA structural polymorphism is of great interest as it may be related to chromosome packing (*1*), gene regulatory processes (*2*−*4*), and the readout of genetic information (*5, 6*). Perhaps the most dramatic expression of this polymorphism is an interconversion between right-handed B-DNA and left-handed Z-DNA. The conformational changes leading to Z-DNA were observed for the first time in 1972 in the CD[1] spectra of poly[d(GC)] recorded in the presence of molar concentrations of sodium chloride (*7*).[2] Crystallographic evidence of a left-handed conformation at atomic resolution was obtained by Wang a few years later (*8*). The correlation between the structure of the crystalline d$(CG)_3$ and the high-salt form of poly[d(GC)] was clarified through laser Raman studies (*9*). Despite intensive studies, the present knowledge on the B−Z transition mechanism is still unsatisfactory. An alternating purine-pyrimidine sequence with a repeat dinucleotide d(GC) unit seems to be necessary, but not sufficient, for such a conversion. There are numerous factors that affect the equilibrium between the right- and left-handed conformations in solution: (1) water activity (*10, 11*); (2) ionic strength (*7, 12*); (3) site-specific interaction with divalent metal cations (*13*−*15*); (4) temperature (*16, 17*) and others (*1, 5, 18, 19*). The B−Z transition is also affected by the chemical modification of DNA, either within nucleobases (*18, 20*−*26*) or within the sugar−phosphate backbone (*1, 27*).

Phosphorothioate analogues of DNA ([PS]-oligos) are useful models for investigating the B−Z transition because of their structural similarity to natural DNA. In these analogues, a sulfur atom is substituted for one of two nonbridging oxygen atoms in the phosphodiester bond. However, this modification creates a center of asymmetry at the phosphorus atom, and a [PS]-oligomer synthesized by a nonstereocontrolled method exists as a mixture of 2^n diastereomers, where *n* is the number of PS bonds. Standard chemical methods for the synthesis of at least partially P-stereodefined oligomers usually employ a dimer block approach (*27, 28*) and provide "chimeric" oligomers (i.e., possessing modified and unmodified internucleotide bonds), with stereodefined phosphorothioate linkages (of R_P or S_P absolute configuration) but only in alternate positions. Studies on "chimeric" oligomers revealed the important consequences of phosphorothioate substitution on the B−Z transition (vide infra). When looking for factors important for the ability of chimeric or fully modified deoxyribonucleoside phosphorothioates to adopt the Z conformation, one should consider differences in an initial B conformation, changes in hydration pattern, altered distribution of charge in phosphorothioate vs phosphate linkages [in phosphorothioate diesters, the charge is predominantly localized at the sulfur atom (*29*)], and changes in interactions with cations. To facilitate the B−Z transition, these factors should either

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[1] Abbreviations: CD, circular dichroism; DMT, 4,4′-dimethoxytrityl; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight (mass spectrometry); PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Because of the method used for sample preparation, the sodium chloride concentrations marked *M* are expressed in moles per liter of solvent.

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Table 1: Melting Temperatures (°C) for Duplexes Formed by Oligonucleotides of $d(CG)_4$ and $d(GC)_4$ Series[a]

type of oligomer	$d(CG)_4$[b]	$d(GC)_4$[b]
unmodified	67	67
unmodified[c]	63	63
[single-S_P-PS]	(**8**)[d] 63	na[e]
[single-R_P-PS]	(**7**) 63	na
[All-S_P-PS]	(**2**) 57	(**4**) 54
[Mix-PS]	52	52
[All-S_PR_P-PS]	(**5**) 52	na
[All-R_PS_P-PS]	na	(**6**) 50
[All-R_P-PS]	(**1**) 50	(**3**) 50

[a] Melting profiles were measured at an oligonucleotide concentration of 6.3 μM (50 μM per nucleotide) with a temperature gradient of 0.2 °C/min in buffer containing 10 mM Tris-HCl (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$, unless otherwise stated. [b] Estimated error ±0.5 °C. The values were obtained from three independent measurements using the first-order derivative method, and differed by less than 1 °C. [c] The buffer used (20 mM sodium citrate, 20 mM NaCl, pH 7.3) was identical to that reported by Cosstick and Eckstein (*27*). [d] The numbers in parentheses are the compound numbers. [e] na: not available.

destabilize the B form or stabilize the Z form. The crystal structure of the chimeric hexamer [R_P-PS]-d[$G_{PS}C_{PO}G_{PS}$-$C_{PO}G_{PS}C$], which was crystallized from a solution of low salt concentration, provided evidence that the molecule exists in the B conformation (*30*). Although the strands differed in the quality of stacking, these changes could not be attributed to the presence of sulfur atoms in the internucleotide bonds, as similar BI and BII conformations were found in CGCG regions of the unmodified dodecamer d(CGCGAATTCGCG). It was also found that phosphorothioate and phosphate linkages were conformationally equivalent. No significant differences in the initial B conformation were found for chimeric [R_P-PS]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$], compared to unmodified oligomer, whereas the substitution is crucial for the B−Z transition of corresponding octamers. It therefore seems that structural factors are more important at stabilizing either the final Z form or the intermediary conformations leading to the final product. Since fully modified stereoregular phosphorothioate oligomers of defined configuration at any selected internucleotide bond became available only recently, as the novel oxathiaphospholane method for their stereocontrolled synthesis was developed (*31*−*33*), there is limited knowledge about their structure and the forces stabilizing it. Therefore, our analysis is based on interactions identified for B and Z structures of unmodified or partially modified oligomers, although one cannot exclude that for fully modified phosphorothioate analogues, important differences with respect to factors stabilizing given structures may exist.

In this report, we discuss the influence of the absolute configuration of P atoms in internucleotide bonds on the ability of stereodefined phosphorothioate oligonucleotides of the [PS]-$d(CG)_4$ and [PS]-$d(GC)_4$ series to adopt the Z conformation. For that purpose, we have synthesized stereodefined, fully modified [All-R_P-PS]-$d(CG)_4$ (**1**) and [All-S_P-PS]-$d(CG)_4$ (**2**), [All-R_P-PS]-$d(GC)_4$ (**3**) and [All-S_P-PS]-$d(GC)_4$ (**4**), [All-S_PR_P-PS]-$d(CG)_4$ (**5**) and [All-R_PS_P-PS]-$d(GC)_4$ (**6**) oligomers, and two octamers modified in only one position, [single-R_P-PS]-d[$C_{PO}G_{PS}C_{PO}G_{PO}C_{PO}G_{PO}C_{PO}G$] (**7**) and [single-$S_P$-PS]-d[$C_{PO}G_{PS}C_{PO}G_{PO}C_{PO}G_{PO}C_{PO}G$] (**8**). All these oligomers are depicted in Table 1. These selected patterns of absolute configurations were intended to answer, at the molecular level, the question about interactions between phosphorothioate centers and other parts of the oligonucleotides during the B−Z transition induced by high sodium chloride concentrations.

MATERIALS AND METHODS

Sodium chloride and Tris base (both of Aristar quality) were purchased from BDH Laboratory, England. Magnesium chloride *pro analysi* was obtained from Merck, FRG. Poly-[d(GC)] (sodium salt) was purchased from Sigma, St. Louis, MO. For the titration of Tris base, hydrochloric acid (amino acid analysis grade reagent, Applied Biosystems, Inc., Foster City, CA) was used.

Chemical Synthesis of Oligonucleotides. The synthesis of unmodified oligonucleotides and [Mix]-oligo(nucleoside phosphorothioate)s was performed on an ABI 380B DNA synthesizer (Applied Biosystems, Inc.) at a 1 μmol scale using a standard phosphoramidite or phosphoramidite/sulfurization protocol, respectively.

The octamers **7** and **8** modified in only one position, and synthesized as a mixture of diastereoisomers, were separated (after detritylation) by means of RP-HPLC (ODS Hypersil column, 250 × 4.6 mm, 10.4% acetonitrile in 0.1 M triethylammonium bicarbonate, isocratic). Enzymatic analysis with snake venom phosphodiesterase and nuclease P1 showed that fast eluting isomer has the R_P configuration and its slow eluting counterpart has the S_P absolute configuration.

The synthesis of stereodefined [PS]-oligonucleotides was performed on an ABI 391 synthesizer. The first nucleoside units were anchored to the solid support by a sarcosinyl linker (*34*). Appropriately protected deoxyguanosyl and deoxycytidyl monomers possessing the 3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure diastereomers. The protocol for the synthesis has been published elsewhere (*32*), and a detailed cycle printout is available upon request.

All synthesized oligomers were purified by two-step RP-HPLC (DMT-on and DMT-off), and their purity was assessed by polyacrylamide gel electrophoresis.

Sample Preparation and Melting Profile Recording. The concentration of oligomers was determined spectrophotometrically by the UV absorbance at $\lambda_{max} = 256$ nm in water, using the extinction coefficient 9900 M^{-1} cm^{-1} for the $d(CG)_4$ sequence (*35*). The samples were then lyophilized and redissolved in 10 mM Tris-HCl buffer (pH 7.0) containing the desired amounts of NaCl. All absorption measurements were carried out in a 1 cm path length cell with a UV/VIS 916 spectrophotometer equipped with a Peltier thermocell (GBC, Dandenong, Australia). Melting profiles were measured at oligonucleotide concentrations of 6.3 μM (50 μM per nucleotide) in a buffer containing 10 mM Tris-HCl (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$ (buffer A) and a temperature gradient of 0.2 °C/min. The melting temperatures were calculated using the first-order derivative method.

Titration Experiments Monitored by UV. During titration experiments, UV spectra were recorded at room temperature. The samples were dissolved in 1 mL of 10 mM Tris-HCl/0.5 M NaCl (pH 7.0). Then, at each step, 29 mg (0.5 mmol) of solid sodium chloride was added, and the samples were

kept at room temperature for 20−60 min (to reach equilibrium) before the next UV spectrum was recorded. The addition of a total 290 mg of NaCl to 1 mL (initial volume of the sample) results in ca. 10% increase of the sample volume at 5 *M* NaCl, but this does not affect the concentrations expressed in moles per liter of solvent (*M*), as it is used in this work. Consecutive spectra were recorded at salt concentrations ranging from 0.5 to 5.0 *M* with a 0.5 *M* increment.

Titration Experiments Monitored by CD. CD spectra were recorded with a CD6 dichrograph (Instruments SA Jobin Yvon, Longjumeau, France) at room temperature using 0.5 cm quartz cuvettes and a nucleotide concentration of 50 μM (6.3 μM per octamer), as this concentration permitted parallel UV measurements. The samples were prepared as described in the previous paragraph. The initial NaCl concentrations were 0 or 0.1 M, and then the consecutive concentrations of NaCl were the same as for the titration monitored by UV (vide supra). Because changes in oligonucleotide concentration caused by the increase of total sample volume were small, the nucleotide concentration of 50 μmol/L was used for calculation of $\Delta\epsilon$ within a whole range of salt concentrations. This slightly changed the observed intensities of CD bands, but did not affect the analysis of patterns of changes in the shape of spectra.

RESULTS AND DISCUSSION

Thermal dissociation studies showed (Table 1) that the duplexes formed by unmodified $d(CG)_4$ and $d(GC)_4$ have melting temperatures of 67 °C in a buffer A, and 63 °C in 20 mM sodium citrate, 20 mM NaCl, pH 7.3 (buffer B). However, when we applied a method based on numerical fitting of melting curves (*36*) for determination of the melting temperature of unmodified $d(CG)_4$, we found the values 64 °C in buffer A and 58 °C in buffer B, rather than 67 and 63 °C, respectively. The value 58 °C is close to the value 55 °C, reported in the literature (*27*), which was obtained in the same way. The identity and purity of our samples were confirmed by MALDI-TOF mass spectrometry (Voyager-Elite, PerSeptive Biosystems Inc., Framingham, MA; negative ion mode, *m*/*z* 2411 M^-, 100%; *m*/*z* 2449 $M^{2-}+K^+$, 30%) and PAGE analysis.

The duplex formed by [All-S_P-PS]-$d(CG)_4$ is thermodynamically more stable than that of [All-S_P-PS]-$d(GC)_4$ (57 vs 54 °C) and is also the most stable among the phosphorothioate analogues. Its melting temperature is 10 °C lower than that for the corresponding natural oligomer. It should be emphasized that the melting temperature of 50 °C found for the oligomer [All-R_PS_P-PS]-$d(GC)_4$ (with alternate configurations) is identical with that for the [All-R_P-PS]-$d(GC)_4$ oligomer, although there are three internucleotide linkages of S_P configuration. The melting temperature for [All-S_PR_P-PS]-$d(CG)_4$ containing four S_P linkages is slightly higher (52 °C). The melting temperatures within other pairs of oligomers are the same, and, since they exceeded 50 °C, all CD measurements were performed at room temperature at which the stability of the self-complementary duplexes was assured. The shapes of the melting profiles were similar for all phosphorothioate oligomers.

Before our results on the B−Z transition of synthesized stereodefined oligomers are presented, a more detailed discussion of literature data is necessary. In the next paragraphs, descriptions such as 5′-*O*-[PS]-dG refer to a phosphorothioate linkage attached to the 5′-oxygen atom of a deoxyguanosine unit.

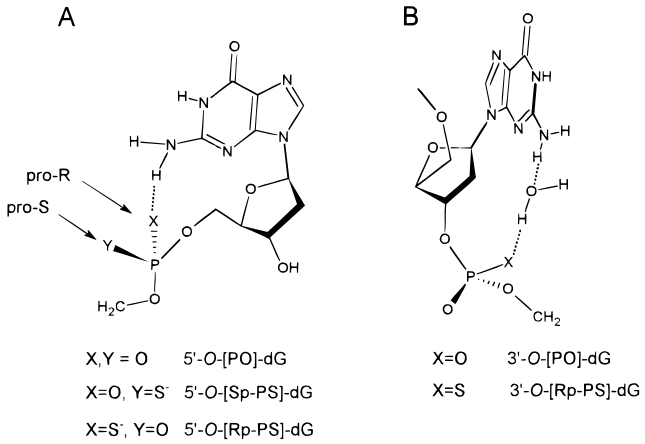

FIGURE 1: Schematic diagrams showing the hydrogen bonding (dotted lines) during initial rotation of the guanine ring (panel A) or formation of the water bridge stabilizing the Z conformation (panel B). Descriptions such as 5′-*O*-[PO]-dG or 3′-*O*-[PS]-dG refer to a phosphate or phosphorothioate linkage attached to the 5′- or 3′-oxygen atom of a deoxyguanosine unit.

Effect of the 5′-O-[PS]-dG at the 3′-End of Chimeric d-[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$]. In the case of short oligomers (less than 12 nucleotides), the 3′-end nucleotide was found to be an important factor affecting the B−Z transition. For example, unmodified $d(CG)_4$, but not $d(GC)_4$, undergoes the B−Z transition with a midpoint of transition at 3 M NaCl (*27*). It is proposed in the literature that, during initial rotation of the 3′-end guanine residue, the *pro-R* oxygen atom marked as X of the phosphate moiety 5′ to the dG residue (Figure 1A, X, Y = O) forms a hydrogen bond with the amino group in position 2 of the guanine (*37*). Most likely, such hydrogen bonding takes place at consecutive guanine residues when conformational change moves along the chain. This bond is charge-assisted (*38*), or, more accurately, a half-charge-assisted, because in phosphate diesters the charge is evenly distributed between both unbridging oxygen atoms X and Y. In chimeric [S_P]-d[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$], where X = O and Y = S^-, the B−Z transition takes place; however, the midpoint of transition at 4 M NaCl suggests that this bond may be slightly less effective, presumably because of reduced electron density around the oxygen atom. As a result, the interaction $-N^2-H-\cdots O{=}P-S^-$ is weaker, but still strong enough to facilitate the nucleation as well as the rotation of consecutive guanine rings.

For [R_P]-d[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$], as well as [$R_P$]-poly[$d(C_{PS}G_{PO})_nC_{PS}G$], the B−Z transition is completely blocked, presumably because it is the sulfur atom in the phosphorothioate linkage (5′ to the dG residue) now forming the hydrogen bond $-N^2-H-\cdots{}^-S-P{=}O$ (Figure 1A, X = S^-, Y = O). Due to the dominant presence of the negative charge at the sulfur atom, this bond should be considered full-charge-assisted, and this strong interaction may prevent the guanine ring from completing its rotation into the syn conformation. This interpretation is in contrast to the common belief that an uncharged sulfur atom (*39*), or a charged sulfur atom in a phosphorothioate moiety (*40, 41*), acts as a hydrogen bond acceptor remarkably less effective

than an oxygen. One could argue that the rotation is incomplete due to a too weak hydrogen bond, rather than because of a too strong hydrogen bond. However, dithiophosphinic acids crystallize as H-bonded dimers (*42*). Also X-ray analysis of a thiolate salt revealed a very strong S−H- - -$^{-}$S hydrogen bond (3.454 vs 4.35 Å calculated for pure van der Waals contacts) (*43*), and theoretical calculations provided bond enthalpies for symmetrical S- - -H- - -S bonds of ca. 60 kJ/mol and S- - -S distances of 3.5 Å (*44*). These data show that, despite the comparatively diffuse character of the acceptor electron pair, an ionized sulfur atom is able to form strong hydrogen bonds. Moreover, a single-site-modified 15-nucleotide RNA hairpin binds 20-fold tighter to MS2 coat protein than the unmodified RNA when the *pro-*R_P oxygen atom of internucleotide linkage involved in hydrogen bonding with an amido group in the side chain of AsnB55 was replaced with a sulfur atom (*41*). When a sulfur atom was substituted for the *pro-*S_P oxygen atom that does not interact with the asparagine side chain, the binding was only 1.6 times strenghtened. This 20-fold enhancement in binding, resulting from the formation of a P−S^{-}- - -H−N bond, was the largest one observed for the set of 13 single-site-modified (both R_P and S_P) oligomers, with modified centers involved in ionic or hydrogen bonding interactions with the protein. Since, in the next paragraphs, we provide evidence for relatively strong water bridges formed with participation of a sulfur atom in a phosphorothioate internucleotide bond, it seems to be more accurate to consider a charged sulfur atom to be a very potent hydrogen bond acceptor.

Effect of the 3′-O-[R_P*-PS]-dG in Chimeric [*R_P*]-d[*$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$*].* It is known that in the Z structure, the oxygen atom (X) of the phosphate moiety 3′ to the dG residue (Figure 1B) forms a water bridge with the amino group in the 2 position of the guanine (*13*). Natural $d(GC)_4$ does not convert into the Z form, most likely, because there is no 3′-terminal dG, which is important for nucleation. Since chimeric [R_P]-poly[$d(G_{PS}C_{PO})_nG_{PS}C$] (*1*) and [R_P]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$] both convert into the Z form, while [S_P]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$] does not, it seems that the presence of the R_P phosphorothioate linkage 3′ to the dG residue [in the R_P-d($G_{PS}C$) unit] stabilizes the Z structure strongly enough to enforce the conversion. To us this also suggests that the water bridge formed with participation of [O═P−S^{-}] is stronger than that formed with [O−P−O]$^{-}$, as more localized negative charge results in an energetically favored interaction. Presumably, unmodified phosphate linkages in the $C_{PO}G$ units also play a positive role, as they may facilitate rotation of consecutive guanine residues along the oligonucleotide chain.

To a certain extent, the observed effect may come from the fact that a sulfur atom has a bigger ionic radius (1.84 Å) than an oxygen (1.32 Å); therefore, the distance between a nitrogen atom at position 2 of a guanine residue and a sulfur atom of a phosphorothioate linkage of R_P configuration is smaller than the 6.1 Å found for the unmodified counterpart (*13*). As the distance of 6.3−6.4 Å is considered the biggest to be spanned by a water bridge (*10*), the bridging in an unmodified compound takes place in the range close to its upper limit, while in the phosphorothioate analogue with the R_P configuration that distance is shorter and the interaction may be more effective.

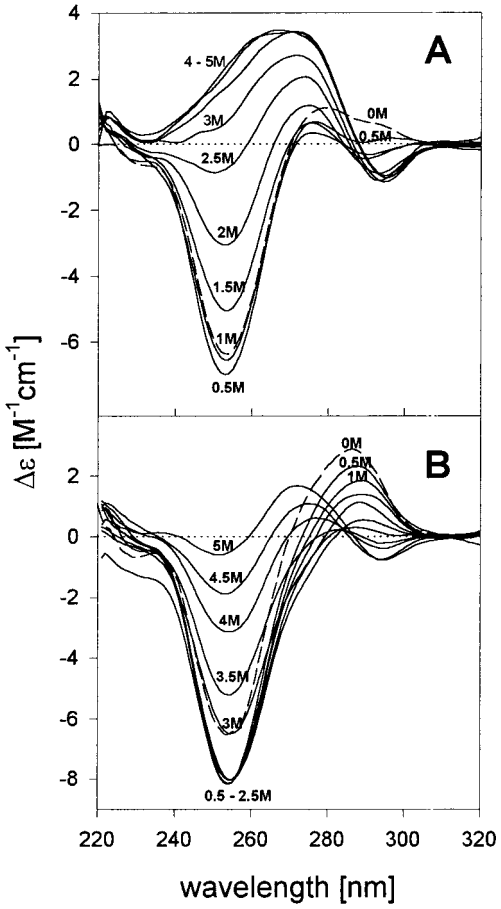

FIGURE 2: CD spectra of [All-S_PR_P-PS]-d$(CG)_4$ (panel A) and [All-R_PS_P-PS]-d$(GC)_4$ (panel B) recorded at NaCl concentrations ranging from 0 M to 5.0 *M*.

These two structural motifs facilitating rotation of the guanine ring and enhancing the water bridge stabilizing the Z form were taken into account in designing the stereodefined phosphorothioate oligomers **5** and **6** with alternate configurations of the P atoms.

NaCl-Induced B−Z Transition of Stereodefined Oligomers with Alternate Configurations of P Atoms. Among stereodefined oligomers, [All-S_PR_P-PS]-d$(CG)_4$ should possess the best programmed composition of structural factors facilitating the B−Z conversion, provided that the above considerations are true. This is because: (1) there is the dG residue at the 3′-end of the oligomer, which is necessary for prompt nucleation; (2) the nucleation is not hampered as phosphorothioate linkages 5′ to the dG residues are of S_P configuration; (3) phosphorothioate linkages 3′ to the dG residues are of R_P configuration, which should stabilize the water bridges. These predictions are confirmed by CD spectra (Figure 2A) and a plot of the absorbance ratio A_{295}/A_{256} against NaCl concentration (*7*) (shown in the Figure 3, closed circles), which provide strong evidence that **5**, upon increasing salt concentration, converts into the Z form. The transition is complete at 3.5 *M* NaCl as the spectra recorded at 3.5, 4.0, and 4.5 *M* are virtually identical. Notably, a midpoint of transition at ca. 2 *M* NaCl, which is 1 *M* lower than that for unmodified $d(CG)_4$ oligomer (ca. 3 *M*, Figure 3, open circles), indicates that **5** undergoes the B−Z transition easier than the natural compound. Comparison of the CD spectra for the Z forms of **5** and unmodified $d(CG)_4$ (both recorded at 4.5 *M* NaCl) reveals their close similarity in the short-wavelength region

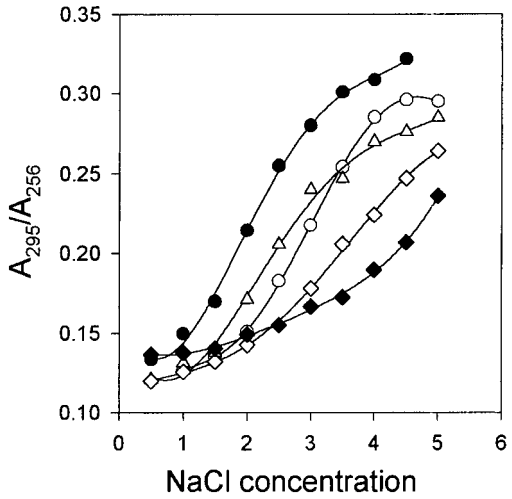

FIGURE 3: Effect of NaCl concentration on the absorption ratio A_{295}/A_{256} in the UV spectra of [PO]-d$(CG)_4$ (open circles), [All-S_PR_P-PS]-d$(CG)_4$ (closed circles), [All-R_PS_P-PS]-d$(GC)_4$ (closed diamonds), [single-S_P-PS]-d[$C_{PO}G_{PS}C_{PO}G_{PO}C_{PO}G_{PO}C_{PO}G$] (open diamonds), and [single-R_P-PS]-d[$C_{PO}G_{PS}C_{PO}G_{PO}C_{PO}G_{PO}C_{PO}G$] (triangles).

(positive maxima at 267 nm, $\Delta\epsilon = 3.4$ and 2.6, respectively), although there are differences in crossover points (288 vs 282 nm) and in the intensities of negative bands at 295 nm ($\Delta\epsilon = -1.1$ vs -2.4).

This outstanding ability of **5** to adopt the Z-DNA conformation supports the assumption that the water bridge formed with participation of the sulfur atom of the phosphorothioate linkage of R_P configuration at the 3′ position of the dG residue significantly facilitates the transition. It was further confirmed by observation that the oligomer **7**, which has only one phosphorothioate internucleotide bond, converts into the Z form (relevant CD spectra not shown) with a midpoint of transition at ca. 2.5 *M* NaCl (Figure 3, triangles), which again is less than for unmodified d$(CG)_4$ oligomer. Notably, its S_P counterpart **8** is 50% converted into the Z form at 3.5 *M* NaCl (Figure 3, open diamonds). Since in both octamers only one internucleotide bond has been modified, so actually they differ in the location of only two atoms (unbridging oxygen and sulfur), one can exclude changes in the hydration pattern as a possible factor affecting the transition. This observation also confirms an earlier suggestion that the conversion starts at the ends of the oligomer (*7*). Presumably, the initial rotation of the guanine residue at the 3′-end of the octamer is identical for both oligomers, and then different stabilization of the Z form by the R_P- or S_P-phosphorothioate moiety located on the opposite strand either facilitates or impedes the conversion of the next segment.

In the next oligomer with alternate configurations of the P atoms, i.e., [All-R_PS_P-PS]-d$(GC)_4$, the 5′-end dG residue has the 3′-*O*-phosphorothioate moiety of favorable R_P configuration, while all other dG's are flanked with favorable 3′-*O*-[R_P-PS] and at least "acceptable" 5′-*O*-[S_P-PS] linkages. Although there is no 3′-end dG residue and typical nucleation is not possible, the 5′-*O*-[S_P-PS]-dG linkages may facilitate the rotation of guanine residues along the oligonucleotide chain in a way similar to that observed for $C_{PO}G$ units in chimeric [R_P]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$] (vide supra), rendering possible the whole process of the B−Z transition. As discussed above, the ability of 5′-*O*-[S_P-PS]-dG linkages to facilitate the rotation of the guanine rings is not as good as that of natural phosphate groups. Therefore, we expected that 50% conversion should occur at a higher salt concentration than the 3.5 *M* NaCl required for chimeric [R_P]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$] (*27*). Indeed, the relevant CD spectra recorded at consecutive NaCl concentrations ranging from 0 to 5 *M* (Figure 2B) showed the pattern of changes very much similar to that observed for **5**. However, even at 5 *M* NaCl the transition was incomplete, and a plot of the absorbance ratio A_{295}/A_{256} (see Figure 3, closed diamonds) confirmed the conversion to the extent of 55%. This value has been calculated by comparison of the observed total increase of this ratio (0.10) with that for **5** (0.19). In the case of the latter compound, an increase of the absorbance ratio value by 0.1 was found for a salt concentration of 2.5 *M*. Notably, the CD spectrum for **6** at 5 *M* NaCl and that for **5** at 2.5 *M* are almost identical. Taking into account the following observations: (1) the transition is not possible to any extent for the natural, unmodified octamer d$(GC)_4$, (2) [All-R_PS_P-PS]-d[$G_{PS}C_{PS}G_{PS}C_{PS}G_{PS}C_{PS}G_{PS}C$] converts into the Z form at higher salt concentration than chimeric [R_P]-d[$G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C_{PO}G_{PS}C$], and (3) [All-$R_P$-PS]-d[$G_{PS}$-$C_{PS}G_{PS}C_{PS}G_{PS}C_{PS}G_{PS}C$] does not convert into the Z form at all, one can conclude that the properties of both internucleotide bonds flanking the dG residues are important for the overall ability to adopt the Z conformation by oligomers of the d$(GC)_4$ series. These data support our hypothesis that, if the typical nucleation at the ends of the duplex is not possible (because of lack of the 3′-end guanosine), the conversion occurs only if the rotation of guanine rings is not hampered (5′-*O*-[PO]-dG or 5′-*O*-[S_P-PS]-dG and not 5′-*O*-[R_P-PS]-dG) and if stronger stabilization of the Z form with participation of the 3′-*O*-[R_P-PS]-dG may take place.

NaCl-Induced Conformational Changes of Stereoregular [All-R_P-PS]-Octamers. The above considerations can be applied to discuss results obtained for the [All-R_P-PS]-d$(CG)_4$ oligomer. Its low-salt CD spectra (Figure 4A) unequivocally indicate the B-DNA family. However, modifications in their shape caused by increasing salt concentrations differ from the pattern for **5**. To follow these changes, the CD difference spectra were obtained by subtraction of the spectrum recorded at 0.1 M NaCl from those recorded at consecutive NaCl concentrations ranging from 1 to 5 *M*. It was previously reported that the B−Z transition is accompanied by a shift of the negative maximum in the CD difference spectrum from about 280 to about 291 nm. Also, the isochroic point (in the difference spectra seen as the point where $\Delta\Delta\epsilon = 0$) moves from 264 nm to longer wavelengths; 275 nm in the case of [All-R_P-PS]-poly[d(GC)] with the midpoint of transition at 2.6 *M* NaCl (*1*). We observed such shifts in the curves starting from 2.5 *M* NaCl (Figure 5A). However, as the isochroic point only moved to 270 nm, it seems that the transition was incomplete. A plot of the absorbance ratio A_{295}/A_{256} against NaCl concentration (shown in the inset to Figure 4A) confirms the presence of a cooperative transition starting above 2 *M* NaCl. Notably, the total increase in the absorption ratio is ca. 50% of that for **5**.

The amounts of the Z form were also assessed numerically based on CD spectra recorded at NaCl concentrations of 1.0−5.0 *M* with a 0.5 *M* increment. All CD spectra were collected for the wavelength range 230−320 nm (with 1 nm increments), digitally filtered and aligned at $\Delta\epsilon$(310 nm) = 0.

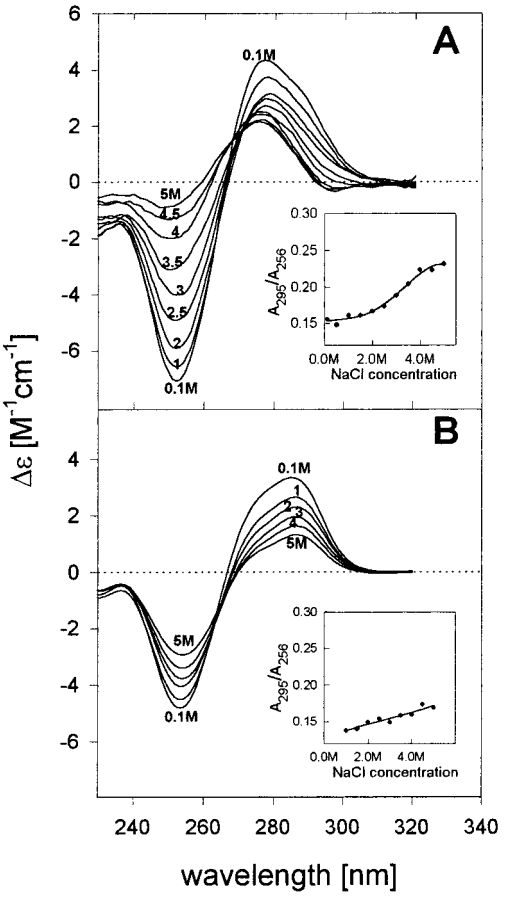

FIGURE 4: CD spectra of [All-R_P-PS]-d$(CG)_4$ (panel A) and [All-R_P-PS]-d$(GC)_4$ (panel B) recorded at NaCl concentrations ranging from 0.1 to 5.0 *M*. The insets show the effect of NaCl concentration on the ratio A_{295}/A_{256} in the UV spectra.

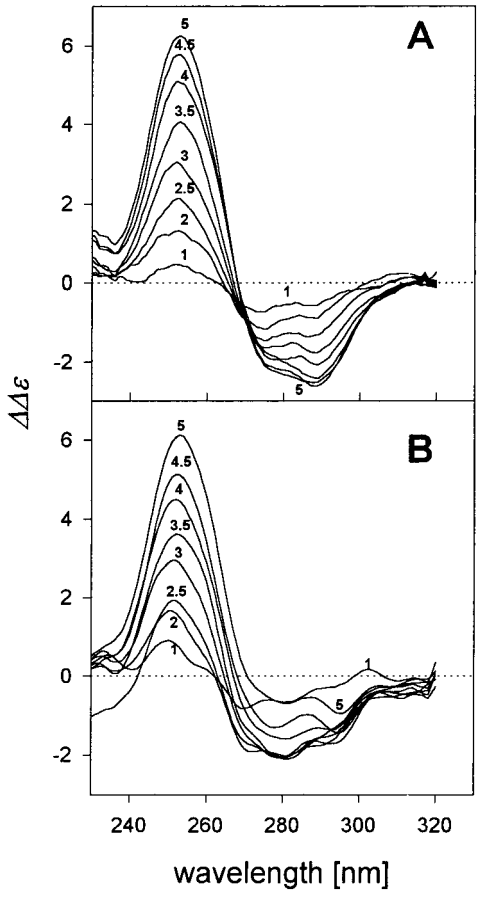

FIGURE 5: CD difference spectra of [All-R_P-PS]-d$(CG)_4$ (panel A) and [All-S_P-PS]-d$(CG)_4$ (panel B) obtained by subtraction of the spectra recorded at 0.1 M NaCl from consecutive CD spectra recorded at NaCl concentrations ranging from 1.0 to 5.0 *M*.

Filtered $\Delta\epsilon$ values ($\Delta\epsilon_{exp}$) were then fit with the formula: $\Delta\epsilon_{calc} = f \times (\%B/100 \times \Delta\epsilon_B + \%Z/100 \times \Delta\epsilon_Z)$, where $\Delta\epsilon_{calc}$ is a value calculated for each wavelength, *f* is a correction factor to eliminate the effect of oligonucleotide concentration error, *%B* and *%Z* are the fractions of B and Z form, respectively ($\%B + \%Z = 100$), and $\Delta\epsilon_B$ and $\Delta\epsilon_Z$ are corresponding values from the model B and Z curves, respectively. The pure Z form component was represented by the CD spectrum of **5** at 5 *M* NaCl. In calculations at consecutive salt concentration steps, we used as the B components the spectra recorded at 0.5 *M* lower salt concentrations; e.g., the spectrum at 4 *M* was simulated using the spectrum recorded at 3.5 *M* NaCl. It was assumed that the changes in the CD spectra associated with that relatively small increase of the salt concentration predominantly result from the increase in the Z form content, rather than from the changes in the spectra of the B form. Within this approach, the calculated values of the %*Z* parameter represent increases in the Z form between consecutive salt concentrations (different by 0.5 *M*), rather than an absolute percentage of the Z form. Recurrent summation gave a result of ca. 50% of the Z form at 5 *M* NaCl, which is close to the value obtained from the plot of the A_{295}/A_{256} ratio against NaCl concentration. This incomplete transition may be explained in terms of the unfavorable effect of 5′-*O*-[R_P-PS]-dG for the nucleation, which is partially overridden by the strong favorable effect of phosphorothioate linkages of R_P configuration 3′ to the dG residues.

Figure 4B illustrates the influence of increasing salt concentration on the CD spectra of [All-R_P-PS]-d$(GC)_4$. The pattern of changes caused by NaCl is the same as for the unmodified oligomer, which does not convert into the Z form. Also a plot of the absorption ratio (shown in the inset to Figure 4B), which remains almost constant, indicates that there is no B−Z transition. It seems that in this case, the favorable effect of 3′-*O*-[R_P-PS-]-dG does not compensate for the lack of the 3′-end dG residue necessary for the nucleation.

[All-S_P-PS]-d$(GC)_4$ and -d$(CG)_4$ at Low NaCl Concentration Do Not Exist in a Typical B-DNA Conformation and Do Not Convert into the Z Structure. The oligomer [All-S_P-PS]-d$(GC)_4$ contains 3′-*O*-[S_P-PS]-dG, i.e., of the opposite configuration to that able to form the strong water bridges. Also there is no 3′-end dG residue necessary for the nucleation. Both these factors should disallow for the conversion. The CD spectrum of **4** recorded at 0.1 M NaCl (Figure 6A) differs from the corresponding spectrum for **3** as the positive band is much lower ($\Delta\epsilon = 1.7$ vs 3.7) and the crossover point is moved to 280 nm, compared to 265 nm for the [All-R_P]-isomer. In consecutive spectra, the positive peak gradually disappears, but the negative one at 254 nm remains rather strong ($\Delta\epsilon = -5$). The absorption ratio (shown in the inset to Figure 6A) is almost constant. This confirms that, as expected, **4** cannot be driven into the Z-DNA form using NaCl. Notably, the spectra recorded at NaCl concentrations as low as 1 *M* are similar to those for

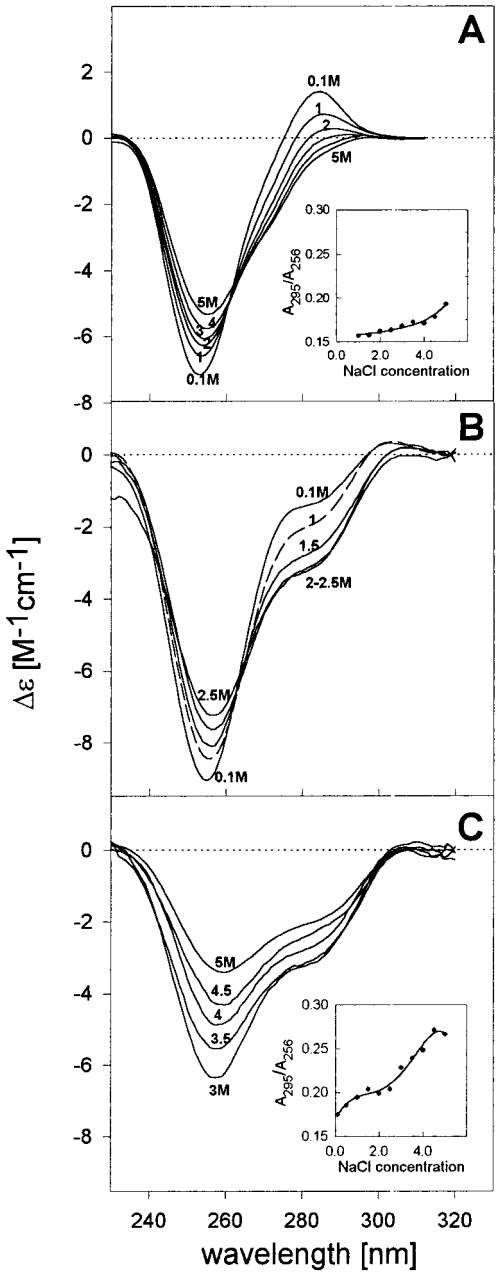

FIGURE 6: CD spectra of [All-S_P-PS]-d$(GC)_4$ (0.1−5.0 *M* NaCl, panel A) and [All-S_P-PS]-d$(CG)_4$ (0.1−2.5 *M* NaCl, panel B; 3.0−5.0 *M* NaCl, panel C). The dashed line in panel B is virtually identical to that reported by van de Sande et al. (*16*) recorded for poly[d(CG)] in the presence of $CoCl_2$. The insets show the effect of NaCl concentration on the ratio A_{295}/A_{256} in the UV spectra.

native DNA at much higher ionic strength (7 M LiCl, 5.5 M NH_4Cl, 6 M CsCl) (*45*) or for DNA fibers/films of low humidity (*46*). An even more distorted spectrum was recorded for the [All-S_P-PS]-d$(CG)_4$ oligomer at 0.1 M NaCl. (Figure 6B). The spectrum is inconsistent with a right-handed helix of the B type at low salt concentration, as there is no positive 270−285 nm band and the presence of a negative shoulder up to 298 nm is noted. Numeric analysis (using PeakFit ver.3.18 software, Jandel Scientific) revealed that this shoulder results from the presence of a negative band centered at 282 nm which modifies the region of 270−300 nm. Therefore, it is impossible to determine whether the positive band at 280 nm, expected for the B-DNA conformation, is present or not. Consecutive spectra at an NaCl concentration ranging from 0.1 M to 5 *M* are shown in Figure 6, panels B and C. The plot of the A_{295}/A_{256} ratio against NaCl concentration, shown in the inset, is different from those for unmodified d$(CG)_4$ and **1**. It consists of two S-shaped segments, and it is unclear if it reflects the B−Z transition. Also, changes in the CD difference spectra (Figure 5B) do not provide any evidence that **2** converts to any measurable extent into the Z form in the presence of NaCl. This suggests that the 3′-*O*-[R_P-PS]-dG motif stabilizing the Z form, as present in alternate compound **5**, is crucial for B−Z transition of fully modified phosphorothioate analogues.

We searched for the reason of observed distortions in the CD spectra of [All-S_P-PS]-oligomers **2** and **4**. In the report by van de Sande et al. (*16*), we found a CD spectrum recorded for poly[d(CG)] in the presence of $CoCl_2$, which was almost identical to that for **2** at 1 *M* NaCl (Figure 6B, a dashed line). Van de Sande assigned this spectrum to an intermediate between the B and Z forms, which is stabilized by cobalt or nickel ions at submillimolar concentration, and can be driven into the left-handed conformation either by heating or by an increase of the Co^{2+} or Ni^{2+} concentration up to 1.5 mM. Studies on the interactions between cobalt(II) or nickel(II) cations and nucleophilic centers in poly[d(CG)] revealed (*14*) that these metal ions stabilize the syn geometry of the dG residue by interactions with the nitrogen atom at position 7 of guanine. If the above-mentioned identity of the CD spectra reflects actual conformational similarity, the mechanism of stabilization must be different, as in our experiment no Co(II) cations were present.

CONCLUSIONS

Fully modified, stereodefined phosphorothioate oligomers [PS]-d$(CG)_4$ and [PS]-d$(GC)_4$ can adopt the Z conformation, provided that the phosphorothioate linkages have appropriate absolute configurations along the oligonucleotide chains, i.e., S_P and R_P at 5′- and 3′-positions of dG's, respectively. The results presented in this report strongly support our hypothesis that two structural factors facilitating the B−Z transition identified for unmodified compounds of d$(CG)_n$, as outlined in Figure 1, play analogous roles for the transition of phosphorothioate analogues. Interestingly, in the case of the [All-S_P-PS]-oligomers **2** and **4**, the stereoregularity of internucleotide bonds resulted in a rather strange initial conformation, as judged by CD spectroscopy. These facts emphasize the important role of stereochemistry of phosphorothioate internucleotide bonds for tuning of physicochemical and, potentially, biological properites of modified oligonucleotides. Our results also provide evidence for the critical importance of water bridges formed with participation of phosphorothioate internucleotide linkages, most likely due to the negative charge being predominantly localized at the sulfur atom.

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