Effect of a single 3′-methylene phosphonate linkage on the conformation of an A-DNA octamer double helix

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ABSTRACT

The three-dimensional structure of the self-complementary DNA octamer d(GCCCG*p*GGC) has been determined in the crystalline state using X-ray diffraction data to a nominal resolution of 2.12 measured from a very small crystal at DESY, Hamburg. The structure was refined with stereochemical restraints to an R value of 17.1%. d(GCCCG*p*GGC), containing one single 3′-methylene phosphonate linkage (denoted *p*), forms an A-DNA double helix with strict dyad symmetry, that is distinct from canonical A-DNA by a wide open major groove and a small average base-pair inclination against the helix axis. The conformation of the unmodified control d(GCCCGGGC) is known from an X-ray analysis of isomorphous crystals (Heinemann et al. (1987) *Nucleic Acids Res.* 15, 9531 – 9550). Comparison of the two structures reveals only minor conformational differences, most notably in the pucker of the reduced deoxyribose. It is suggested that oligonucleotides with charged 3′-methylene phosphonate groups may form stable duplexes with complementary DNA or RNA strands rendering them candidates for use as gene-regulatory antisense probes.

INTRODUCTION

Chemical modifications of the phosphodiester backbone of DNA exert an influence on the physical properties and the conformation of the macromolecule (1). With regard to three-dimensional structure it must be investigated to which extent the replacement of a phosphodiester linkage with a phosphorothioate, a phosphotriester, a phosphoramidate, a methyl or a methylene phosphonate group disturbs DNA duplex conformation. At present, the knowledge in this field is limited to studies of mono- and dinucleotide analogs. On the oligonucleotide level, only one crystal structure analysis of a phosphorothioate-substituted DNA hexamer duplex has been reported (2).

Recently, backbone-modified oligodeoxyribonucleotides have received much attention for their potential usefulness as antisense probes to block replication, transcription or translation of certain viral or cellular target sequences. Oligonucleotides with methylphosphonate linkages have been employed (3–8) to circumvent problems, inherent in this approach, of limited uptake and short half life of antisense oligonucleotide in the target cell and of limited affinity to the target sequence (9). In general, results have been promising: The inhibition of human immunodeficiency virus (HIV) replication in cultured cells by methylphosphonate-substituted oligonucleotides has been more effective than with unmodified control sequences (10).

Whereas uncharged methylphosphonate groups offer the advantage of enhanced membrane permeability and nuclease resistance, the reduced solubility of the resultant oligonucleotides in aqueous buffers (10) limits their usefulness as antisense DNA. Furthermore, methylphosphonate-substituted oligodeoxynucleotides bind to target DNA single strands with lower affinity than unmodified controls unless hybridization takes place under very low salt conditions (11). The destabilization arises predominantly from methylphosphonate groups with pseudo-axial stereochemistry (12).

Conversely, achiral and charged methylene phosphonates isoelectronic with phosphodiester groups can be generated when either deoxyribose $O_{5'}$ or $O_{3'}$ atoms are formally replaced with a methylene group (Figure 1). Here we report the chemical synthesis of a self-complementary DNA octamer, d(GCCCG*p*GGC), where the *p* denotes a 3′-deoxy-3′-methylene phosphonate, and the crystal structure analysis of the resultant duplex. Comparison with the crystal structure of the unmodified octamer d(GCCCGGGC) (13) shows that the introduction of isolated 3′-methylene phosphonate linkages has a very small effect on DNA helix conformation.

EXPERIMENTAL PROCEDURES

DNA synthesis was carried out in analogy to procedures described elsewhere (14) by the modified triester approach in solution. The octanucleotide was assembled by consecutive elongations with dimeric blocks starting from the 3′-end. The dimer block bearing the internal 3′-methylene phosphonate linkage was synthesized

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from a protected 3'-deoxy-3'-methylene phosphonyl guanosine as triethylammonium salt (15) and a traditional G-OH component. Detritylations throughout the synthesis were achieved using zinc bromide solutions (16). Intermediates were purified over silica gel columns or plates. After final deprotection using oximate, ammonia or acetic acid, respectively, the product was purified over a Mono Q 10/10 column (Pharmacia-LKB) using a sodium chloride gradient in 10 mM NaOH pH 12, the peak fractions being desalted over a C_{18} cartridge. UV melting curves recorded in 20 mM sodium cacodylate pH 7 at 12 μM strand concentration show the T_m values of d(GCCCG*p*GGC) and of the unmodified analog to be identical within experimental limits, 44°C *versus* 45°C.

Single crystals were grown at 4°C by microdialysis (17) of 8.3 mM DNA single strand in 10 mM Tris-HCl pH 7.0, 10 mM magnesium acetate against the same buffer solution containing 5% (v/v) 2-methyl-2,4-pentanediol (MPD). Small truncated tetragonal bipyramids were obtained which were shown by X-ray precession photography to be isomorphous with crystals of the native octamer d(GCCCGGGC) duplex (13). The space group is $P4_32_12$ and unit cell constants are a = 43.30 Å and c = 24.52 Å, suggesting an octamer single strand to be the content of the asymmetric unit. Despite numerous attempts these crystals could not be grown large enough for data collection using in house X-ray sources.

A 0.12×0.12×0.04 mm crystal was used for data collection with the image plate system developed at the EMBL outstation at DESY, Hamburg (Hendricks and Lenfter, unpublished). At the X/31 beam line in HASYLAB, employing a wavelength of 1.040 Å and a crystal-to-plate distance of 202 mm, data could be collected out to a nominal resolution of 2.12 Å by oscillating the crystal on an Arndt-Wonacott camera in 4° increments around the a-axis for a total range of 104°.

The diffraction data were processed with the MOSCO software (18) yielding an agreement factor of 9.7% (on intensities) for the data set. Structure calculations were performed with 1303 (1σ) structure amplitudes in the 6–2.12 Å resolution range. This data set is essentially complete in the high-resolution range but lacks about 30% of the theoretically observable reflections between 6 and 4 Å resolution which were flagged as overloads. The mean fractional isomorphous difference between the 1051 structure amplitudes observed in both the present data set and the data set of the unmodified (native) octamer duplex (13) was 13.8% after scaling by a modification of the method of Fox and Holmes (19) using an anisotropic temperature factor. The good agreement between diffraction data sets and unit cell parameters of both oligonucleotides justifies the use of the three-dimensional structure of the unmodified octamer d(GCCCGGGC) as a starting model for refinement.

The stereochemically restrained least-squares structure refinement (20,21) as implemented in the program NUCLIN (22) was carried out essentially as for other DNA structures determined in this laboratory (13,23–25). Restraints on torsion angles were not applied and water molecules were selected by stereochemical criteria from the 20 highest peaks of difference electron density maps visualized on a vector graphics screen with FRODO (26). When refinement of the input model had reached an R value of 20%, atom $O_{3'}$ of residue G(5) showed several non-ideal bond length and bond angle distances as well as the highest temperature factor of all intrachain $O_{3'}$ atoms. At this point $O_{3'}$ was replaced with a methylene group and refinement was continued until convergence with restraints for the methylene phosphonate linkage taken from a model X-ray structure (27). Details concerning diffraction data and restrained refinement are summarized in Table 1.

RESULTS

Refinement results

Structure refinement was terminated when the difference Fourier map was essentially featureless showing a maximum density of 0.242 eÅ^{-3} and only one additional peak over 0.2 eÅ^{-3} which could not be assigned as solvent positions. After the last cycle of refinement, the rms positional and thermal shifts were 0.007 Å and 0.16 Å^2, respectively. The final model comprising 161

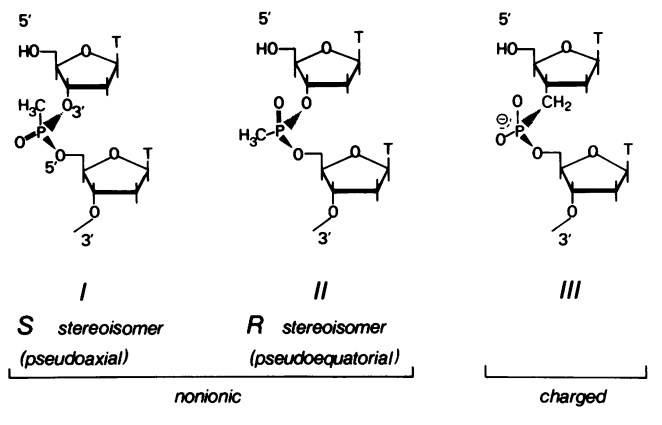

Figure 1. Chemical structures of three different methyl and methylene phosphonate linkages in DNA. d(GCCCG*p*GGC) contains one 3'-deoxy-3'-methylene phosphonate (*p*) of type III.

Table 1. Refinement statistics.

Resolution range (Å)	6.0–2.12
Number of observations (1σ on F_o)	1303
(3σ on F_o)	1197
R factor (1σ data) (%)	17.1
(3σ data) (%)	16.0
F_o/F_c correlation coefficient	0.957
$\Sigma F_o/\Sigma F_c$	1.003
$<\|F_o-F_c\|>$	7.74
Sugar/base bond distances (Å)	0.015 / 0.025
Sugar/base bond angle distances (Å)	0.035 / 0.050
Phosphate bond distances (Å)	0.055 / 0.050
Phosphate bond angle distances and H-bond distances (Å)	0.074 / 0.075
Planar groups (Å)	0.020 / 0.030
Chiral volumes (Å^3)	0.072 / 0.100
Single torsion non-bonded contacts (Å)	0.132 / 0.250
Multiple torsion non-bonded contacts (Å)	0.208 / 0.250
Isotropic thermal factors (Å^2)	
Sugar-base bonds	3.13 / 5.00
Sugar-base bond angles	3.92 / 7.50
Phosphate bonds	4.81 / 7.50
Phosphate bond angles, H-bonds	4.37 / 7.50
Structure amplitudes	/ 4.50

F_o and F_c are observed and calculated structure amplitudes, respectively. The R factor is $\Sigma|F_o-F_c|/\Sigma F_o$ and the correlation coefficient is $\Sigma[(F_o-<F_o>)(F_c-<F_c>)]/[\Sigma(F_o-<F_o>)^2\Sigma(F_c-<F_c>)^2]^{1/2}$. For stereochemical parameters, the left number gives the deviation from ideal values in the final model and the right number is the target variance used in refinement. The weight applied to the corresponding restraint is the inverse square of the target variance.

atoms for an octamer single strand and 29 water oxygens with unit occupancy yields an R value of 17.1%. Electron density of the oligonucleotide is contiguous and generally well defined, but density in the solvent region is not as clear as might be expected due to a number of strong, low-angle reflections missing in the data set. Coordinates and X-ray structure amplitudes for d(GCCCG*p*GGC) have been submitted to the Brookhaven Protein Data Bank.

Comparison with d(GCCCGGGC)

In general, the conformation of the 3′-methylene phosphonate-substituted octamer is very similar to that of the unmodified DNA duplex. After a least-squares superposition the rms distance between equivalent atoms in the two structures is 0.29 Å. The mean error in atomic coordinates according to Luzzati (28) is between 0.15 and 0.2 Å for both structures. Since this estimate attributes all differences between observed and calculated structure amplitudes to errors in the model, thus representing an upper limit for the average coordinate error, the overall deviation between the two structures is small, but significant. The carbon atom of the 3′-methylene phosphonate linkage deviates from the corresponding $O_{3'}$ of the unmodified octamer by 0.56 Å.

Global helix structure

As its unmodified counterpart, d(GCCCG*p*GGC) forms a double helix the two strands of which are related by perfect crystallographic dyad symmetry (Figure 2). Helicoidal parameters of the molecule were determined with the NEWHEL90 program (29,30) and are given according to the Cambridge conventions (31). Bases on the the first strand are numbered G(1) through C(8) from 5′ to 3′ and bases of the symmetric, self-complementary strand G(9) through C(16).

The helix is of the A type as evident from the large average displacement of base pairs from the helix axis of 3.85 Å and the predominance of $C_{3'}$-*endo* sugar puckers (see below). Analysis with the program CURVE (32,33) shows that the helix axis is essentially straight. The average helix rotation of 31.1° (30.4°) gives 11.6 (11.8) base pairs for a complete turn of the helix (numbers in parentheses are for the native octamer). An unusually small average inclination angle of 7.5°, a mean rise

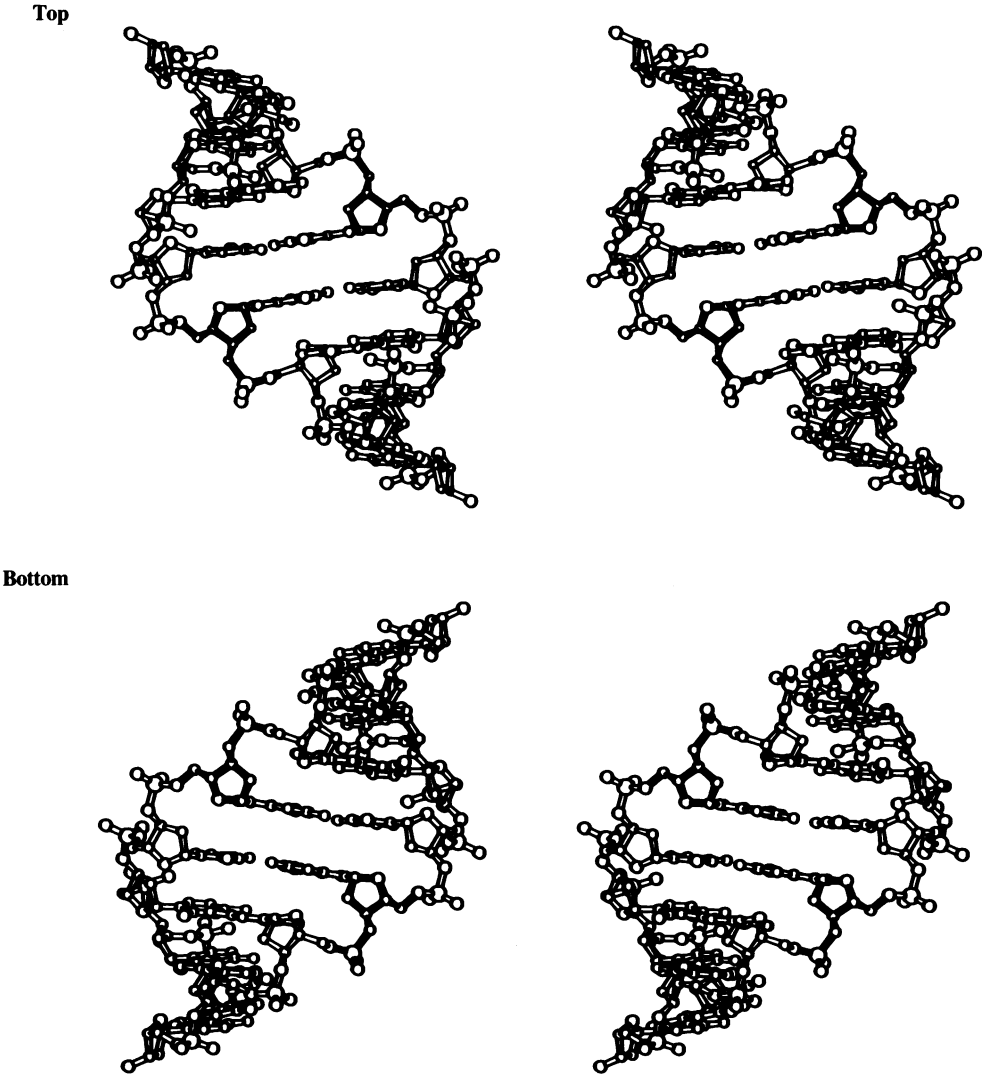

Figure 2. Stereo drawing of the A-DNA octamer d(GCCCG*p*GGC). The view is into the major groove of the symmetric duplex (top) and into the the minor groove (bottom). The sugar-phosphate portion of G(5) bearing the 3′-methylene phosphonate is drawn with solid bonds.

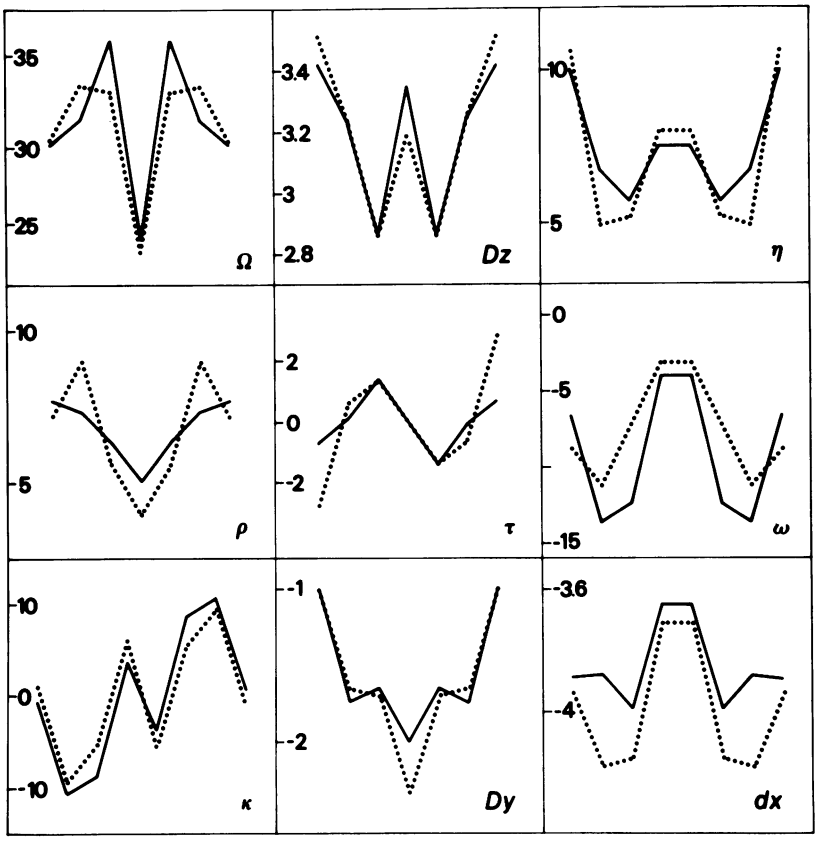

Figure 3. Variation of important helical parameters in d(GCCCG*p*GGC) (solid lines) and in d(GCCCGGGC) (13) (dotted lines). Twist (Ω), rise (Dz), inclination (η), roll (ϱ), tilt (τ), propeller twist (ω), buckle ($\varkappa$), slide (Dy) and x displacement (dx) are plotted against the sequence. Rotational parameters are given in degrees, translational parameters in Å.

Table 2. Backbone torsion angles.

	α	β	γ	δ	ϵ	ζ	χ
G1			58(48)	85(79)	−148(−161)	−67(−56)	−162(−159)
C2	−94(−85)	172(162)	75(69)	77(71)	−160(−160)	−64(−63)	−161(−162)
C3	−78(−61)	180(175)	51(42)	93(81)	176(168)	−64(−46)	−153(−159)
C4	−57(−82)	171(−174)	36(54)	86(81)	−170(−178)	−74(−69)	−151(−160)
G5	151(−171)	−177(−164)	−179(143)	*96*(68)	*−156*(−155)	*−73*(−73)	−172(−177)
G6	*−72*(−78)	175(−178)	62(57)	69(80)	−155(−148)	−66(−75)	−166(−173)
G7	−51(−69)	167(174)	45(62)	84(79)	−158(−162)	−72(−67)	−160(−166)
C8	−48(−50)	166(167)	45(56)	108(146)			−143(−120)
Av.[a]	−66(−71)	174(177)	53(55)	84(86)	−162(−165)	−69(−64)	−159(−160)
SD[a]	18(14)	6(12)	13(9)	9(25)	12(15)	4(10)	9(17)

Torsion angles are given between −179° and 180° and are denoted according to the IUPAC-IUB recommendations (39). Torsion angles involving the phosphonate methylene group are printed in italics. The numbers in parentheses refer to the unmodified octamer d(GCCCGGGC) (13). [a]Average and standard deviation excluding the torsion angles α and γ of G(5) and δ of C(8).

per base pair of 3.1 Å and an average major groove opening of 9.3 Å put the structure into a sub-family of A-DNA helices (34,35) that is quite distinct from canonical A-DNA (36).

The variation of the most important helicoidal parameters with the octamer sequence d(GCCCG*p*GGC) (Figure 3) is very similar as in d(GCCCGGGC). The most prominent local structural feature, the inter-strand G(5)-G(13) purine-purine overlap at the central base-pair step is rather well conserved although there is a tendency towards less extreme values of small twist (Ω) and roll (ϱ) and large magnitude of slide (Dy). Inter-strand guanine-guanine stacking is frequently observed at central C.G base-pair steps in A-DNA octamers (13,37,38).

Backbone conformation

Since d(GCCCG*p*GGC) carries a chemical modification at the backbone, this is where we may expect to see structural perturbations. The backbone torsion angles (Table 2) generally lie within the range expected for an A-DNA helix (1) and do not deviate dramatically from those of the unmodified octamer. Interestingly, the extended all-*trans* backbone of residue G(5), forced by the inter-strand stacking, has undergone a slight crankshaft rotation around the $O_{5'}$-$C_{5'}$ bond (torsion angle β) changing α from −171° to 151° and γ by approximately the same amount from 143° to −179°. All other changes in torsion

Table 3. Deoxyribose conformation.

	τ_M	P	Sugar pucker	
G1	36.9(39.1)	22.5(8.6)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
C2	45.5(43.9)	24.3(21.9)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
C3	37.1(45.0)	30.6(12.8)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
C4	31.6(35.1)	15.6(22.3)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
G5	*28.1(47.8)*	*351.8(16.0)*	$C_{2'}$-*exo*	($C_{3'}$-*endo*)
G6	43.4(41.8)	13.7(18.9)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
G7	38.5(43.2)	16.0(15.3)	$C_{3'}$-*endo*	($C_{3'}$-*endo*)
C8	17.3(42.8)	74.1(186.5)	$O_{4'}$-*endo*	($C_{3'}$-*exo*)
Av.[a]	37.3(42.3)	16.4(16.5)		
SD[a]	6.1(4.1)	12.3(4.9)		

Pseudorotation magnitude τ_M and phase P are given according to (41). Residue G(5) bearing the 3'-methylene phosphonate group is shown in italics. [a]Average and standard deviation excluding the 3'-terminal residue C(8).

Table 4. Crystal contacts between duplexes.

Possible hydrogen bond(s)		Symmetry operator		
$G(6)N_2$ – $G(1)N_3$		$-x+3/2$	$y+1/2$	$-z+3/4$
$G(5)N_2$ – $G(1)O_{4'}$		$-x+3/2$	$y+1/2$	$-z+3/4$
$C(8)O_{2P}$ – W_{03}	– $C(8)N_4$	$-y+3/2$	$x-1/2$	$z-1/4$
$G(7)N_3$ – W_{04}	– $C(8)O_2$	$-y+3/2$	$x-1/2$	$z-1/4$
$G(7)N_3$ – W_{04}	– $C(8)N_3$	$-y+3/2$	$x-1/2$	$z-1/4$
$G(7)N_2$ – W_{04}	– $C(8)O_2$	$-y+3/2$	$x-1/2$	$z-1/4$
$G(7)N_2$ – W_{04}	– $C(8)N_3$	$-y+3/2$	$x-1/2$	$z-1/4$
$C(8)O_{4'}$ – W_{04}	– $C(8)O_2$	$-y+3/2$	$x-1/2$	$z-1/4$
$C(8)O_{4'}$ – W_{04}	– $C(8)N_3$	$-y+3/2$	$x-1/2$	$z-1/4$
$C(3)O_2$ – W_{15}	– $G(1)N_2$	$y-1/2$	$-x+3/2$	$z+1/4$
$C(3)O_2$ – W_{15}	– $C(8)O_2$	$x-1/2$	$-y+3/2$	$-z+5/4$
$C(4)O_{2P}$ – W_{20}	– $C(8)O_{1P}$	$-x+3/2$	$y+1/2$	$-z+7/4$
$C(8)O_{1P}$ – W_{22}	– $C(8)O_{3'}$	$y+3/2$	$-x+1/2$	$z+5/4$
$C(4)O_{2P}$ – W_{23}	– $C(8)O_{3'}$	$-y+1$	$-x+2$	$-z+3/2$

Upper part: Direct hydrogen-bonded contacts between duplexes. Bottom part: Crystal contacts *via* possible duplex-water-duplex hydrogen bonds.

angle values are statistically insignificant with two exceptions. The first concerns the δ torsion angle of G(5) which is related to the pucker of the modified deoxyribose (see below) and the second concerns the 3'-terminal C(8) which displays different sugar pucker and glycosyl angle in both octamers. In d(GCCCG*p*GGC) the C(8) sugar shows above average temperature factors and an unusually small degree of pucker (Table 3) probably indicating some kind of disorder. C(8) is also engaged in many crystal contacts (see below) which may strain its conformation to some extent.

Sugar pucker in A-DNA oligonucleotide structures is preferably $C_{3'}$-*endo* with the possible exception of 3'-terminal sugars which tend to be flexible and ill-defined (40). In d(GCCCG*p*GGC) the reduced deoxyribose of G(5) deviates from standard pucker by adopting $C_{2'}$-*exo* pucker (Table 3) whereas all other sugars except for nucleoside C(8) have standard geometry. This change in sugar pucker is small but significant and represents the most direct structural consequence occurring upon the chemical modification of a phosphodiester to a 3'-methylene phosphonate group in the octamer. Unfortunately, to the best of our knowledge there are no crystal structures of model deoxyriboses with methylene groups replacing $O_{3'}$ against which to check the influence of this modification on pucker as seen in our crystals.

Crystal packing and DNA hydration

Helix-helix contacts in crystal lattices are known to exert a considerable influence on DNA duplex structure (25,42–45). It must be asked, therefore, whether the small differences observed between the structure of d(GCCCG*p*GGC) and its unmodified analog may be caused by packing effects. Since the two octamers crystallized isomorphously, the packing of helices is very similar in both. The phosphonate methylene group does not participate in interhelical contacts of any kind. There are three loose van der Waals contacts with water molecules having carbon-oxygen distances above 3.8 Å which are very unlikely to influence the conformation of the sugar-phosphonate backbone.

As in the crystal structure of d(GCCCGGGC), intermolecular contacts involve almost exclusively minor groove atoms (Table 4). There are only two direct hydrogen bonds between symmetry-related molecules (four per double helix) disregarding the Watson–Crick hydrogen bonds in the duplex that also emanate from crystal symmetry since one strand only constitutes the asymmetric unit. The backbone linking residues G(5) and G(6) does not participate in direct or water-mediated hydrogen bonds; in fact, the site of chemical substitution had been chosen such as to minimize the effect of lattice contacts. Thus, the small changes in helix conformation observed in the crystal structure of d(GCCCG*p*GGC) must be due to the chemical substitution and not to crystal artefacts.

The 3'-terminal residue C(8) is involved in more than half of the contacts listed in Table 4. It is therefore especially sensitive to small changes in crystal packing. This may serve to explain the differences in the conformation of C(8) between the two structures.

In the d(GCCCG*p*GGC) crystal structure, 28 water molecules with $<B> = 42.3$ Å^2 were found per DNA single strand, and 39 waters with $<B> = 42.2$Å^2 were found in d(GCCCGGGC). The larger number of waters around the unmodified octamer is explained by the higher resolution of X-ray data available in that analysis. 11 water molecules occupy equivalent positions in both crystals, i.e. their oxygens are within 0.7 Å from one another and make similar contacts with the DNA. The mean temperature factors of these atoms are 36.7 and 36.5 Å^2, respectively. Thus, the hydration shells of both octamers are similar with respect to the well-defined water positions indicated by below-average B values.

DISCUSSION

The crystal structure of d(GCCCG*p*GGC), a self-complementary DNA octamer with one 3′-methylene phosphonate group, was solved at a nominal resolution of 2.12 Å. X-ray diffraction data were collected from a tiny single crystal using synchrotron radiation at DESY, Hamburg. This demonstrates the feasibility of structure analyses with macromolecular crystals normally considered too small for X-ray data acquisition.

A comparison with the unsubstituted octamer d(GCCCGGGC) (13) reveals only minor differences in atomic positions. Derived helicoidal parameters agree rather well, but small differences are discernible. Inspection of the diagrams displayed in Figure 3 provides a good estimate of how sensitive some of the parameters, routinely used to describe nucleic acid helix structure, are with respect to small changes in atomic coordinates. The characteristic inter-strand guanine-guanine stacking at the central base-pair step of d(GCCCGGGC) is preserved in the present structure, but not quite as pronounced as in the former.

The replacement in d(GCCCG*p*GGC) of the $O_{3'}$ function of G(5) with a methylene group leads to a change in sugar pucker from $C_{3'}$-*endo*, as usually observed in A-DNA, to $C_{2'}$-*exo*. Whether this change is attributable to a stereoelectronic effect in the reduced deoxyribose is difficult to assess in the absence of theoretical work and accurate structural data from model compounds. The altered sugar pucker appears to influence the conformation of the sugar phosphate backbone towards the 5′-side where torsion angles α, β and γ, in the unusual *trans-trans-trans* conformation undergo a crankshaft rotation by approximately 40° relative to the unmodified reference helix. This motion in the backbone is structurally linked with the slightly reduced inter-strand purine-purine stacking at the central base-pair step.

The A-DNA duplex examined here may serve as a model for an RNA-DNA hybrid double helix, the usual product of an antisense oligonucleotide directed against single-stranded RNA (9). RNA-DNA hybrids as well as double-stranded RNA normally form double helices of the A-form (1). With regard to the potential usefulness of oligonucleotides carrying charged methylene phosphonate groups as antisense probes, it must be emphasized that an isolated group of this kind has a very small influence on the conformation of the DNA double helix. It may thus be expected that the stability of hybrid duplexes where one strand contains single 3′-methylene phosphonate linkages is about the same as for unmodified DNA duplexes. The observation that d(GCCCG*p*GGC) has the same melting point as the control sequence lends support to this view. Single substitutions, however, may not be sufficient to enhance the half life of oligonucleotides in living cells, as it is known that certain exonucleases are able to jump over isolated nonionic methylphosphonates, requiring at least two adjacent substitutions for arrest of cleavage (4). Whether several adjacent 3′-methylene phosphonates would destabilize DNA or DNA-RNA hybrid duplex structure by the cumulative effect of small changes in DNA backbone conformation and whether they would give rise to enhanced nuclease resistance remains to be established in future work.

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