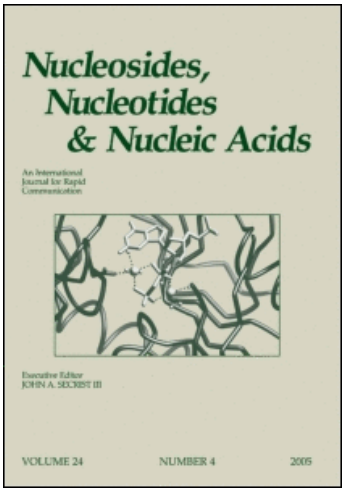

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STRUCTURAL CORRELATIONS OF BACKBONE MODIFICATIONS IN ANTISENSE OLIGONUCLEOTIDE DUPLEXES

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Abstract

This article presents a collective of structural analyses concerning backbone modified oligonucleotides and various factors which correlate the conformational preference of backbone modifications with the stability of antisense duplexes.

Introduction

The concept of gene regulation using antisense principles is based upon the use of oligodeoxynucleotides or their analogs (designated oligonucleotides herein, unless otherwise noted) which target specific biologically important sites within mRNA sequences.[1-5] Conceivably, antisense oligonucleotides may function as a gene blocker against RNA binding enzymes. Another role of antisense oligonucleotides is implicated in their ability to modulate the conformation and topology of the resultant complexes through their binding to target RNA. The formation of antisense complexes induces changes in the conformation of RNA strands and in the surface properties (such as polarity and accessible areas) at or near the binding sites, thereby altering their susceptibility to enzymatic activities. The enhanced rate of RNA cleavage by RNases in the presence of complementary antisense oligonucleotides are exemplary of gene fragmentation *via* antisense mechanisms.

Although promising as effective therapeutics for a number of gene regulatory diseases, antisense oligonucleotides were quickly shown to be unstable in a cellular environment. One of the major drawbacks is backbone cleavage by endo- or exo-nucleases, rendering their half lifetimes as short as a few minutes in serum fluids.[6] The poor resistance to metabolic degradation is clearly detrimental to the effectiveness of antisense oligonucleotides. To extend the survival time of antisense oligonucleotides, a plethora of backbone modifications have been designed and used as inter-nucleotide linkages in the hope that these chemical moieties would inhibit enzymatic cleavage without sacrificing binding affinity.[1-4,7,8] The reported binding properties and stabilities for some of these sequences vary greatly because sequence contexts and experimental conditions are often different. In general, one up to all of the backbone atoms have been substituted but the number of chemical bonds (six rotatable bonds) between two residues is preferably kept constant. The substituent groups include charged and neutral as well as

chiral and achiral types. As compared to natural phosphodiester achiral and reduced charge types are the preferred choices. The representative backbone modifications are phosphorothioate (chiral, charged),[9] formacetal/thioformacetal (neutral, achiral),[10,11] peptide-like (neutral, achiral),[8] carbamate (the morpholino nucleotides[12]) and methylmethyleneimino linkages (neutral, chiral N).[7]

A few promising backbone modifications have been extensively studied using a variety of biophysical methods.[7,13-27] Ideally one would like to find a correlation of chemical and structural modifications with targeted desirable properties. Therefore, a focus of research in this area is to establish a set of rules and a comprehensive structure data base which would permit prediction of antisense properties based on given chemical structures or provide clues to the *de novo* design of antisense molecules. The herein reported NMR studies of backbone modified oligonucleotides are the results of such an effort in our laboratory. Our NMR and UV studies of antisense related oligonucleotides are based on a model dodecamer duplex (Figure 1). Each of the backbone modifications is placed in the center of the strand d(CGCGTT*TTGCGC), so that the modified and the unmodified duplexes can be systematically compared. The complementary strand of the modified sequences can be either DNA to give DNA•DNA duplexes or RNA to give RNA•DNA hybrid duplexes. The latter are models of *in vivo* antisense actions. The comparison between the DNA•DNA and RNA•DNA duplexes should reveal the difference in hybridization requirements. We have reported the detailed studies of the unmodified (DI and RI), the formacetal (FMA in DII and RII) and the 3'-thioformacetal modified (3'-TFMA in DIII and RIII) duplexes using NMR and UV spectroscopy.[21-24,26] The high resolution structure of the RIII duplex has been elucidated.[27] Recently, preliminary results for the methylmethylene imino (MMI in RIV and RV hybrid duplexes) containing duplexes have been obtained.[28]

A Brief Review of Previous Studies

The comparisons of the unmodified DI with the FMA modified DII and the 3'-TFMA modified DIII and the similar comparisons of RI with RII and RIII indicate that the effect of these single backbone modifications on overall helical structure is local, while their impact on duplex stability is only observed at the global level as duplex strands dissociate. The local effect of these backbone modifications is reflected in the small chemical shift differences for the majority of proton resonances and mainly unperturbed phosphorous NMR spectra for duplexes with or without modifications. The inter-proton interactions (NOEs) are of comparable intensities for those of non-linker resonances in unmodified and modified duplexes. However, the differences in sugar and backbone conformations of the linker site (note that each of the synthetic linker contains a pair of methylene protons, providing NMR probes for the linker conformation) are easily discernible.

The above results reveal that the FMA linker, located 3' to the T6 sugar in DII or RII (Figure 1), confers a C2'-endo pucker preference for that residue, while the backbone conformations are close to that of B- or A-form canonical duplexes, respectively. Therefore, the FMA linker should be better accommodated in DNA•DNA duplexes, which typically assume C2'-endo sugar pucker, than in RNA•DNA duplexes. In comparison, the incorporation of an FMA linker into an RNA•DNA duplex, which displays sugar pucker features that reflect an average between C2'- and C3'-endo puckers, suffers from the unwillingness of the linker to adopt such an averaged backbone

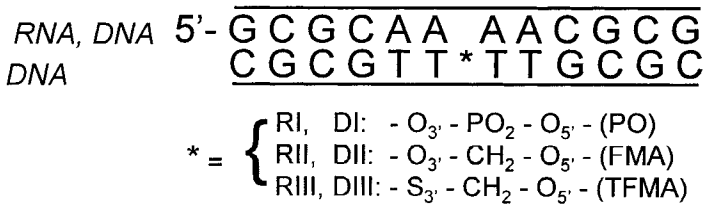

FIG. 1. DNA and RNA sequences used in NMR and UV studies.

conformation. Besides the sugar torsion angles, in either type of duplexes, the FMA linker is present in a conformation that deviates from its lowest energy form as derived from quantum mechanics calculations.[24] These results are consistent with the ~ 3 °C reduction in $T_{m,uv}$ (melting temperature of the duplex as derived from the UV measurements) for DII and RII compared to DI and RI, respectively, suggesting that sugar conformation preference may not be determinant among the various factors stabilizing the duplexes containing FMA linker modifications. Otherwise, a larger T_m reduction would have been observed for the RII duplex compared to the DII duplex.

The 3'-TFMA linker, which differs from the FMA linker in the 3'-substitution with an S atom, behaves differently compared to the FMA linker.[22,26] This linker, located 3' to the T6 sugar in DIII and RIII, confers that residue a preference for a sugar pucker that is more C3'-endo like. The most prominent feature of this backbone linker, however, is its adoption of significantly different conformations in the DIII and the RIII duplexes. To illustrate this point, the three dimensional (3D) structures of the linker segment in the two duplexes are shown in Figure 2 and a line plot of the backbone torsion angles is provided in Figure 3.

In DIII, the presence of a larger S atom at the 3'-position forces a C3'-endo type sugar pucker (see δ angles in Figure 3). This adjustment is accompanied by major deviations of the ε, ζ and α angles from the values of canonical phosphodiester torsion angles in A- or B-form duplexes (Figure 3).

The TFMA linker in DIII is structurally unstable, shown by linker methylene proton signal line broadening at lower temperatures. The DIII duplex exhibits a lower T_m, compared to that of the FMA modified DII, as measured by UV (by 1 °C) and NMR temperature dependent experiments. The unusual conformation of the DIII linker derived from NMR has been confirmed by theoretical modeling calculations to be one of the meta-stable forms.[24] When the TFMA modified DNA strand is hybridized with the RNA complementary strand, the backbone distortions observed in DIII are alleviated and the backbone geometry is restored to a much more A-form like geometry (Figure 3). In contrast to the DII and DIII comparison, the RIII duplex is slightly more stable than the FMA modified RII. However, the stabilization effect is rather small, even though there are large amplitude variations in the backbone conformations of the two duplexes (Figure 3).

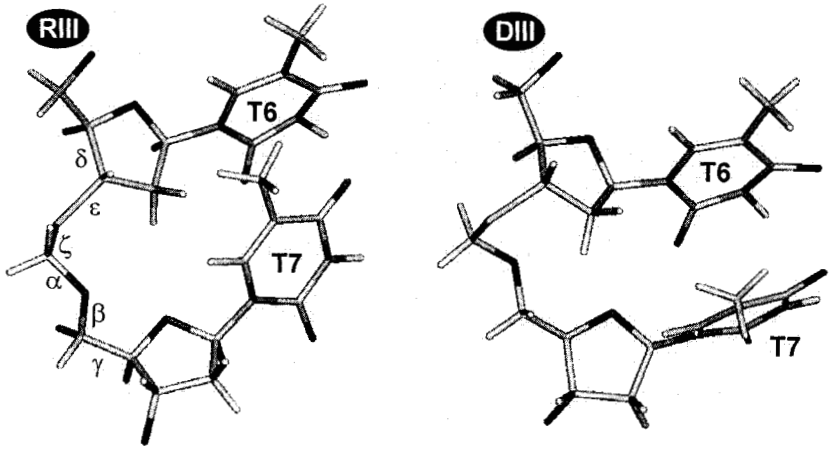

FIG. 2. Structures of the 3'-TFMA dimer in RIII[27] and DIII[25] duplexes.

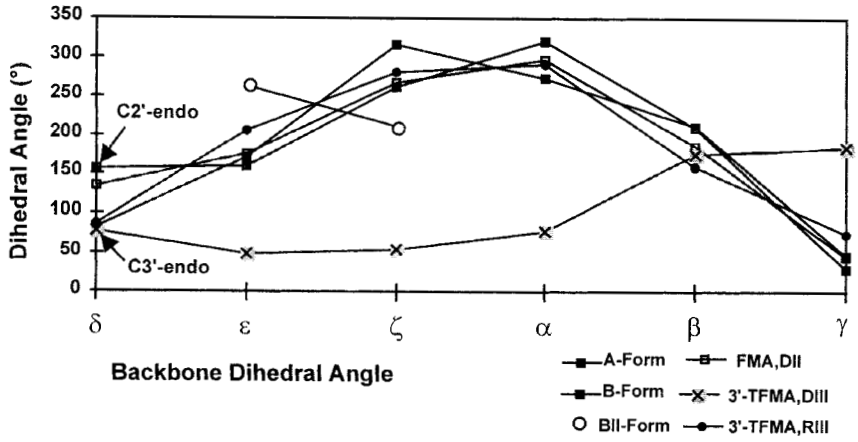

FIG. 3. Backbone torsion angle comparisons of backbone modified duplexes with canonical A- and B-form duplexes [parameters were obtained from the model structures generated using the QUANTA program (Molecular Simulations Inc.)]. Alternative BII-Form of DNA differs from B-Form in ε and ζ angles as shown.

A Survey of the Factors Affecting Backbone Conformations and their Correlations to Duplex Stability

The studies of a single incorporation of linker modifications into a cassette molecule have revealed detailed conformations of FMA and 3'-TFMA in DNA•DNA or RNA•DNA duplexes.[21-24,26,27] These results demonstrate the intricate interplay of various factors, such as the first principles of bonding geometry, steric interactions, and the polarity of molecular surfaces, and offer a broader view on the structural and stability effects of various chemical modifications. In this discussion, based upon our studies and the available literature information,[13-27] the correlations of chemical modifications with backbone conformations and duplex stability will be explored. By comparisons of backbone conformations at the modification sites, common features that are of critical concern in designing new backbone modifications will be brought to attention.

(a) The effects of 3'-substitution

In addition to our studies of 3'-O-CH_2- (FMA) and 3'-S-CH_2- (TFMA) substitutions in backbone moieties of oligodeoxynucleotides, 3'-CH_2-PO_2-,[17] 3'-CH_2-NMe-,[29] and 3'-NH-PO_2-[18] in deoxyribonucleotides and 3'-CH_2-SO_2-[30] in a ribonucleotide dimer as backbone linkages have been examined using crystallographic and NMR spectroscopic methods. These studies demonstrate that except for the 3'-O-CH_2- substitution, all substitutions mentioned above favor averaged C3'-endo like sugar pucker conformations. This conformational transition occurs regardless their differences in neighboring residues or paring complimentary strands. Furthermore, these 3'-subtitution atoms are linked to groups of different chemical nature (CH_2, PO_2, NMe, or SO_2). Thus, these C3'-substituents may vary in polarity and hydrophobicity, but are common in that they are all bulkier groups compared to the natural form, 3'-O. These analyses support the notion that changes in sugar pucker are predominantly driven by the size of the 3'-substituted atoms or steric factors, rather than electronic configurations (the gauche effect). A C3'-endo arrangement shifts 3'-substituted atom to an equatorial-type position, releasing the steric interactions between this atom (or group) and other sugar atoms (Figure 4). In analyzing the 3'-O-CH_2- substitution, an increased contribution of C2'-endo sugar pucker as compared to that of unmodified DNA sugar ring is discernible. For this group, it seems that neither electronic gauche nor steric effect can satisfactorily explain the experimental observations. Sugar moieties are more rigid as compared to backbone bonds, and thus, the prescribed sugar pucker preferences of 3'-substitutions have a profound effect on the conformation of backbone frames (*vide infra*).

(b) The inherent conformational preference of backbone linkers

Quantum mechanics, molecular mechanics and dynamics simulations have been applied to investigate the conformational preference of several backbone linkers.[24,25,29,31] Theoretical studies of model molecules for the FMA and TFMA linkers demonstrate that each of the molecules studied prefers a low energy conformation, which is different from that assumed by canonical DNA or RNA.[24,25] Indeed, when the linker structures derived

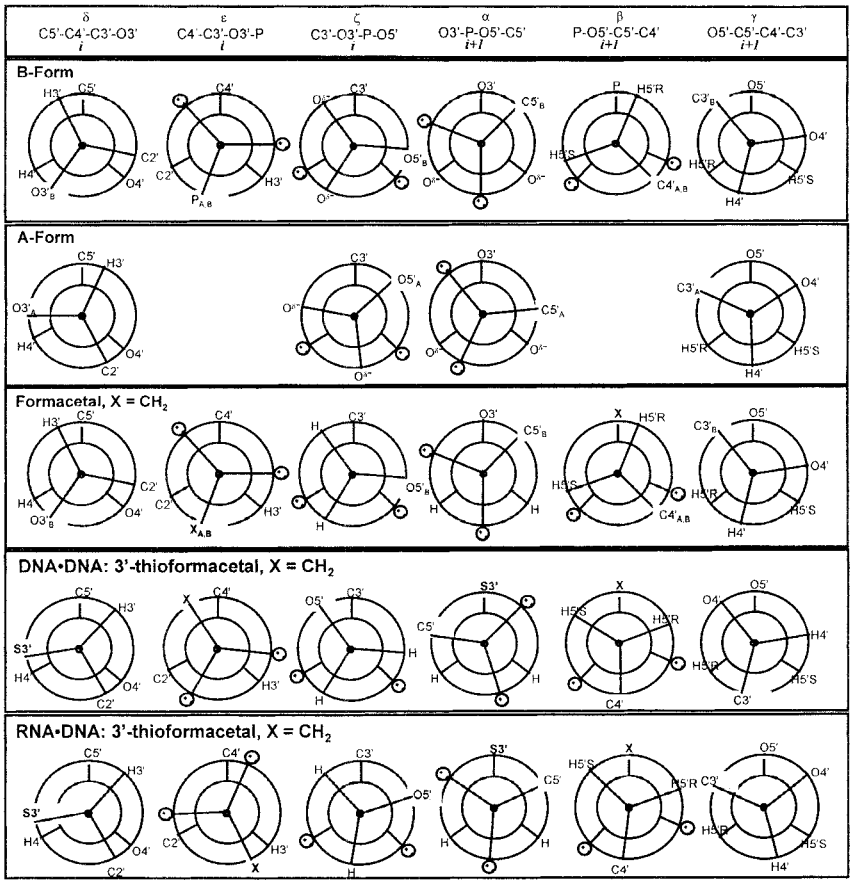

FIG. 4. Newmann Projections of the six backbone angles linking T6 and T7 residues. The drawings show relative orientation of the polar and non-polar groups and the outlay of the backbone surfaces. The lone pair electrons of oxygen atoms are shown as circles with two dots.

from NMR experiments are compared with those derived from the ideal models, the disagreement between them is evident. Moreover, the energy cost due to linker conformational change is reflected in the stability of the duplexes. The larger the discrepancy between the ideal and actual structures, the less stable the duplexes are. For instance, the 3'-TFMA linkage has been shown to prefer a trans-torsion angle for C-C-C-S by quantum mechanics.[25] This model angle corresponds to a trans C1'-C2'-C3'-S (υ_s) angle in a deoxyribose ring, which is consistent with a C3'-endo sugar pucker (υ_2 ~35° and υ_s ~160°).[32] In DIII, the υ_s angle is somehow reduced because an anti υ_s angle is not quite compatible with the overall helical structure of the DNA•DNA duplex, and the backbone angles are of unusual values. The significant deviations between the theoretical

and actual structures of the TFMA linker in DIII correlates with the most unstable feature of this duplex. At present, there are only limited number of backbone modifications that have been vigorously examined using both theoretical and experimental methods and there is no sufficient information available to unambiguously define the correlations between conformational deviation from ideal values and the stability of duplexes. In this area, further studies are definitely needed in order to sustain the postulation that the naturally evolved phosphodiester linkers are the best optimized templates for designing the lowest energy forms of duplexes.

(c) The self-compensation of backbone torsion angles

To minimize the effect on stable base pairing and stacking, the changes in backbone conformations tend to be inter-related and they compensate each other in a way so that the net change in backbone orientation as a right handed helix is minimal. Several semi-empirical correlation diagrams are available in the literature to describe the correlations between α (the P-O5' torsion) and ζ (P-O3') angles,[33] ζ and ε (O3'-C3') angles,[34] and ζ and δ (C4'-C3') angles.[34] Our results have shown major backbone angle changes in the 3'-TFMA modified DNA•DNA (DIII), and to a lesser degree, in the RNA•DNA (RIII) duplexes. In comparison to model A-form parameters, changes in the backbone parameters (ε, ζ, α and β angles) of RIII show an oscillating pattern (Figure 3) with ε and α angles larger than the ideal values and ζ and β angles lower than the ideal values. The more drastically altered backbone angles of DIII display a pattern in which the ε, ζ and α angles are reduced by 90 - 180° from the ideal values. These variations are only compensated by a higher γ value (Figure 3). As expected from backbone conformation variations, both duplexes, DIII and RIII are less stable than the corresponding unmodified duplexes. RIII is, in comparison, less destabilized with respect to RI (ΔT_m = 2.8 °C) than DIII with respect to DI (ΔT_m = 4.1 °C). These results suggest the detrimental effect of large amplitude structural re-adjustments due to backbone modifications to the thermal stability of the duplex.

The available information on modified backbone torsion angles presents an expanded energy contour map where new families of backbone angles that have never been observed with natural phosphodiester nucleotide linkages exist. Although it is premature to conclude any general rules at the present stage, the results obtained thus far extend our ability to construct the backbone frames for the design of new generations of antisense oligonucleotides.

(d) The hybridization effect

Upon recognition of the complimentary strand (or hybridization), the resultant conformations of the sequences containing the same linker can be markedly different. This reflects the fact that the receptor sequences assert a conformational restraint to the binding sequence. One such example is related to the 3'-TFMA linker in a DNA•DNA duplex[22] compared to that in an RNA•DNA[26] duplex. For the two duplexes, the sequence contexts are identical except for the modified strands: one contains deoxyribose rings as opposed to ribose rings in the other duplex. NMR experiments detected conformational averaging behavior in the 3'-TFMA linker containing DNA•DNA duplex, and therefore demonstrated that the linker is unstable in this duplex. The linker also destabilizes the DNA•DNA duplex more than it destabilizes the RNA•DNA duplex as revealed by comparisons of thermodynamic parameters derived from UV melting profiles. The pattern of the inter-proton interactions (NOEs) of the same linker in the two duplexes are

distinctly different. The modeling calculations and NMR restrained structure elucidation subsequently reveal that the ε, ζ and α backbone torsion angles of the linker in the DNA•DNA duplex are extraordinarily lower in value than those of the RNA•DNA duplex (Figure 3).[26] This direct comparison of linker conformations between the two duplexes clearly demonstrates the influence of the overall structure of the duplex and the possibility that the backbone modifications can re-adjust their conformations to minimize the global energy of the molecule. Thus, a compromise is reached between the intrinsically preferred conformation and the global fitness of the linker moiety. For the 3'-TFMA linker, the backbone distortions are mainly attributed to the strong preference of the 3'-S for a less crowded position. This sugar conformation appears to force the adoption of unusual torsion angles in the rest part of the linker backbone.

(e) The polarity of the backbone surfaces

Protein molecules are often known to organize their structures in hydrophilic and hydrophobic patches consisting of closely located polar or non-polar residues, respectively.[35] This type of arrangements is not as evident in nucleic acids, but it does exist along the helical backbone structures (Figure 4). For instance, the Newmann projections of the α ($O3'_{i-1}$-P_i-$O5'_i$-$C5'_i$, i is the residue number) and ζ ($C3'_i$-$O3'_i$-P_{i+1}-$O5'_{i+1}$) angles of both A- and B-form helices, which are around the phosphate linkage, show in-phase alignment of the negatively charged phosphate oxygen atoms and the lone pair electrons of O5' or O3'. These arrangements result in electron-negative, polar surfaces on one side of the backbone (Figure 4). The backbone linkages away from phosphate, such as γ and δ angles, present more hydrophobic surfaces consisting of sugar carbon and hydrogen atoms (Figure 4). Assuming the ideal backbone geometry, the substitution of PO_2 with CH_2 in FMA and 3'-TFMA would have a major impact on the polar surface alignments around the α and ζ angles with methylene hydrogen atoms in the gauche orientation to the oxygen lone pair electrons. The substitution would also cause the surfaces around the β and ε angles to become more hydrophobic because of the exposed CH_2 group. These variations may not be favored by the assumed conformation upon modification and may partially be the cause of duplex destabilization. Clearly more vigorous surface electrostatic calculations and using better defined model systems are needed in order to understand the hydrophobic and hydrophilic interactions and their correlation to nucleic acid duplex stability.

Conclusion

Antisense oligonucleotides have attracted intense research effort. The success of this approach relies on stable duplex formation of oligonucleotides with target RNA to block specific gene regulation sites or to induce RNA strand cleavage by RNase H at the binding site. To further the progress in this field, it is of fundamental importance to understand the molecular basis of binding affinity and sequence specificity achievable by antisense oligonucleotides.

We summarize here the results of our studies on six oligonucleotide duplexes containing unmodified or backbone modified antisense DNA strands (Figure 1). Using comparisons, we highlight the most relevant properties that contribute to the stability of these duplexes. These comparisons show promising correlations that ultimately will lead to the understanding of the principles that govern the formation of stable duplexes. What we have not addressed here are the sequence dependence and the structural homogeneity requirements. In principle, the factors discussed above should play equally important

roles in duplexes of different sequences. The sequence dependent behavior of a linker may be identified from its enhanced or reduced effects on the overall properties of several duplexes of varied compositions. The structural homogeneity requirement or the effect of multiple substitutions may be examined by comparisons of duplexes containing varied levels of backbone modifications at designated positions. We hope that our continued studies will shed light on these points.

Acknowledgments

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