

# Conformational Preorganization, Hydration, and Nucleic Acid Duplex Stability

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CHEMICAL MODIFICATION OF DNA AND RNA and the generation of artificial oligonucleotide pairing systems have been spurred by two central objectives. Although initially unrelated, both promise new insights into the nature of the double helical conformations assumed by DNA and RNA. The first concerns issues of molecular evolution and, among many others (Breslow, 1998), seeks a rationalization for nature's selection of pentoses over other potential candidates as the carbohydrate building blocks of the genetic material (Eschenmoser and Loewenthal, 1992). Chemical synthesis of alternative nucleic acid pairing systems and an exploration of their physical-chemical properties can potentially yield insights to such questions (Böhringer et al., 1992; Eschenmoser and Kisakürek, 1996). For example, comparison of the conformational and pairing properties of hexose-DNA and DNA has led to the view that the helicity of DNA is a consequence of the inherent geometric constraints of the deoxyriboses in its backbone (the situation in RNA is similar) (Eschenmoser and Dobler, 1992). Furthermore, the Watson-Crick base pairing priorities in DNA duplexes (G-C > A-T) are no longer valid in duplexes formed by (2',3'-dideoxy- $\beta$ -D-glucopyranose)-based oligonucleotides (G-C > A-A  $\approx$  G-G > A-T) (Hunziker et al., 1993). Thus, the standard base pairs in DNA are not simply a consequence of the chemical nature of the four bases, but their formation is also related to the nature of the backbone sugar moiety.

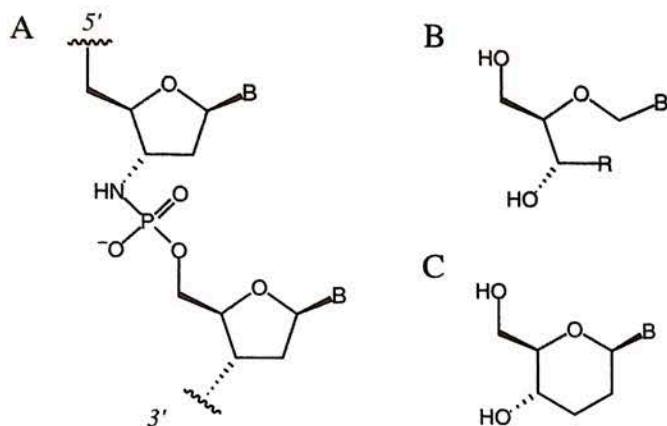
The search for oligonucleotides with sufficient metabolic stability for *in vivo* applications has provided the second strong incentive for chemically modifying DNA and RNA. DNA and RNA oligonucleotides are readily degraded biologically, and in the case of RNA, chemical stability is also insufficient. Chemical modification may render them more resistant to degradation by various nucleases. However, chemical modification should not impair RNA affinity and pairing selectivity. Some 200 modifications were reviewed recently, and their hybridization affinities for complementary RNA were analyzed in the context of the expected structural changes (Freier and Altmann, 1997). This structure-activity correlation has led to the identification of several stabilizing features. Thus, modifications that provide higher RNA affinity either (1) preorganize the backbone in conformations favorable for hybridization to RNA, (2) improve stacking through addition of a polarizable group to the base het-

erocycle, (3) increase the number of hydrogen bonds, for example, replacement of A by 2,6-diaminopurine, (4) neutralize the negative charge of the phosphate group, or (5) feature combinations of (1) to (4). However, incorporation of isolated dimethylene sulfone RNA building blocks (Richert et al., 1996) into oligodeoxynucleotides resulted in a drastic destabilization of the corresponding DNA-RNA duplexes (Bäschlin et al., 1996) despite the preference of the sulfone RNA backbone for an A-type C3'-*endo* conformation and the fact that the negatively charged phosphodiester moiety is replaced with a neutral linker (Roughton et al., 1995; Egli, 1996). This example demonstrates that subtle geometric changes caused by a chemical modification (S-C vs. P-O bond lengths, S-C-C vs. P-O-C bond angles) can offset features that in principle should enhance RNA affinity (Northern-type pucker, neutral backbone).

N3'  $\rightarrow$  P5' phosphoramidate DNA (3'-NP DNA), a second-generation nucleic acid analog in which O3' is replaced with an amino group, displays drastically enhanced RNA affinity compared with DNA and RNA (Fig. 1A) (Gryaznov and Chen, 1994; Chen et al., 1995; Gryaznov et al., 1995). Moreover, 3'-NP DNA self-pairing is considerably more stable than duplex formation in the case of either DNA or RNA. It is noteworthy that stable formation of Pu-Py\*Py-type triplexes with both duplex DNA and RNA and 3'-NP DNA as the third strand was observed at neutral pH, where triplex formation with a phosphodiester third strand does not occur (Escudé et al., 1996). Circular dichroism (CD) experiments suggested that 3'-NP DNA duplexes resemble the RNA A-form (Gryaznov et al., 1995), and 2-dimensional NMR data showed that the 3'-aminofuranoses in 3'-NP DNA duplexes adopt predominantly *N*-type puckers (Ding et al., 1996). 3'-NP DNA oligonucleotides display improved nuclease resistance relative to DNA and RNA, and the accumulated *in vitro* and *in vivo* data suggest that phosphoramidates can serve as potent and specific antisense (Heidenreich et al., 1997; Skorski et al., 1997) and antigene agents (Giovannangeli et al., 1996, 1997) and potentially as decoys (Rigl et al., 1997).

We have recently determined the crystal structure of the 3'-NP DNA duplex with sequence 5'-d(CnpGnpCnpGnpAnpAnpTnpTnpCnpGnpCnpG) at 2 Å resolution (Tereshko et al., 1998). This is the first crystal structure of an all-chemically modified DNA double helix. Consistent with the earlier CD and





**FIG. 1.** Structures of nucleoside and nucleotide analogs. (A) N3' → P5' phosphoramidate DNA (Gryaznov and Chen, 1994; Chen et al., 1995; Gryaznov et al., 1995). (B) Glycerol DNA (R = H) (Schneider and Benner, 1990) and 1',2'-seco-DNA (R = CH<sub>3</sub>) (Peng, 1993). (C) Homo-DNA (Böhlinger et al., 1992; Eschenmoser and Dobler, 1992; Eschenmoser and Loewenthal, 1992; Hunziker et al., 1993).

solution NMR data, the overall conformational features of the 3'-NP DNA duplex in the crystal closely resemble those of double-stranded RNA. Indeed, the structural, chemical, and biological data now accumulated for 3'-NP DNA strongly suggest that this compound behaves more like RNA and, despite its name, has few similarities with DNA. This is important for the discussion to follow, and the reader should bear in mind that although double-helical DNA can also adopt an A-type geometry, the reasons for doing so are entirely unrelated to those that bring about the A conformation with 3'-NP DNA. Pioneering x-ray diffraction studies of DNA fibers conducted by Franklin and Gosling (1953) had shown the molecules to undergo a structural transition when the humidity was lowered below a certain level. We now know that this is the conformational switch between the well-hydrated B-form and the less hydrated A-form duplex. There is overwhelming evidence that isolated DNA duplexes do not adopt the A geometry in solution. Conversely, the adoption of an A-type geometry by double-helical 3'-NP DNA in both solution and the solid state provides evidence that its conformational preference is entirely a consequence of replacing the backbone 3'-oxygen with an amino group.

Although the higher propensity for an A-type geometry displayed by the 3'-NP DNA backbone may not come as a surprise (lower electronegativity of its deoxyribose 3'-substituent), several other characteristics of phosphoramidate DNA are initially puzzling. First, the stability differences between 3'-NP DNA/RNA duplexes and the corresponding phosphodiester duplexes are unexpectedly high [2.3°C–2.6°C per modified linkage (Gryaznov and Chen, 1994; Chen et al., 1995; Gryaznov et al., 1995)]. Second, phosphoramidates with the DNA 5'-oxygen replaced by an amino group pair with neither DNA nor RNA (Gryaznov et al., 1995). Third, combining the 3'-NP DNA backbone with the 2'-O-methyl modification leads to a loss of stability relative to pairing between DNA and RNA, although 2'-O-methyl RNA has higher affinity for RNA than both DNA and RNA (S. Gryaznov, unpublished observations). The crystal structure of the 3'-NP DNA dodecamer duplex pro-

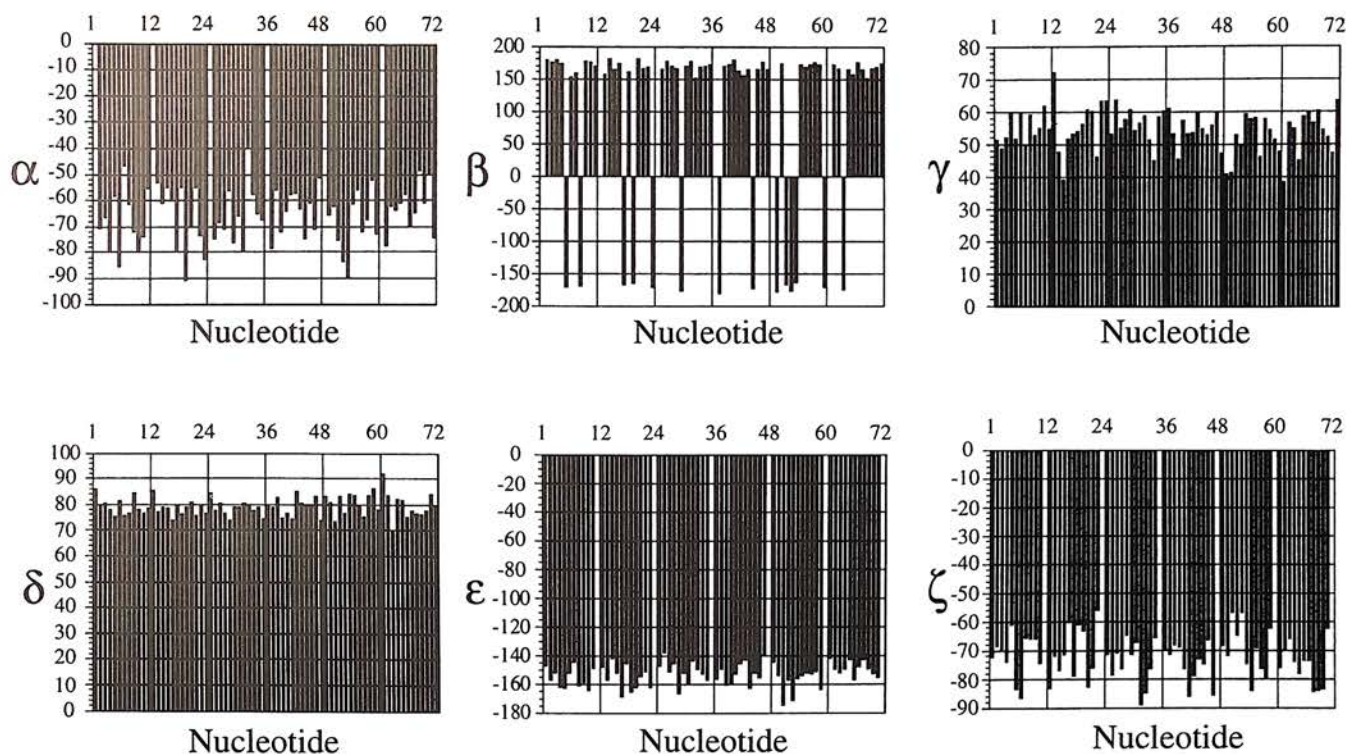
vides qualitative explanations for all of these issues. In particular, the structure hints at three factors, conformational preorganization, stereoelectronic effects, and hydration, as the main sources of the unusual properties of 3'-NP DNA. Here, I highlight the specific contributions by each of these factors to the structure and stability of 3'-NP DNA and discuss their roles in duplex structure and pairing stability in general.

## CONFORMATIONAL PREORGANIZATION

It was shown that oligodeoxynucleotide single strands can exhibit stabilizing intramolecular interactions that enthalpically poise them for duplex formation (Vesnaver and Breslauer, 1991). Similarly, one would expect pairing between oligonucleotide strands that are conformationally preorganized for the duplex state to be entropically favored relative to the formation of a duplex between strands whose conformations in the single-stranded and duplex states differ significantly (Hunziker et al., 1993). Studies of the pairing behavior displayed by oligonucleotide analogs with altered backbone flexibilities support this hypothesis (Griffey et al., 1994). The UV melting temperatures of duplexes containing flexible nucleosides derived from glycerol were lowered by 9°C–15°C per incorporated nucleoside analog (Fig. 1B) (Schneider and Benner, 1990). Although no details for the entropic and enthalpic contributions to this substantial loss in stability were provided, it is likely that the unfavorable entropic term for a pairing between strands with higher flexibility is dominant in this respect. Similarly, 1',2'-seco-DNA oligonucleotides displayed no self-pairing, and mixed pairing between complementary 1',2'-seco-DNA oligonucleotides and DNA oligonucleotides did not occur either (Fig. 1B) (Peng, 1993). These results are consistent with the lower degree of order present in a 1',2'-seco-DNA single strand relative to a DNA single strand. Conversely, pairing between homo-DNA oligonucleotides, an analog with 2'-deoxyribose replaced by 2',3'-dideoxy-β-D-glucopyranose (Fig. 1C), was found to be entropically favored relative to pairing between corresponding DNA strands (Hunziker et al., 1993).

The thermodynamic parameters for pairing between phosphoramidate DNA strands reveal that its high stability of self-pairing is enthalpy based, and the entropy term is considerably less favorable than in the case of either DNA or RNA (Gryaznov et al., 1995; Tereshko et al., 1998). The crystal structure reveals a highly uniform duplex structure and conformational variations in the backbone torsion angles that are smaller than those commonly observed in the crystal structures of DNA and RNA duplexes. Thus, the root mean square (rms) deviations for the individual torsion angles in the 72 nucleotides that constitute the crystallographic asymmetric unit are all around or below 10 degrees (Fig. 2). Further, it is noteworthy that the conformations of all  $\alpha$  and  $\gamma$  torsion angles fall into the  $-sc$  (synclinal) and  $+sc$  ranges, respectively. In A-form DNA and RNA duplexes, one commonly finds an extended backbone variant, with both  $\alpha$  and  $\gamma$  assuming an *ap* (antiperiplanar) conformation. These findings provide evidence that the 3'-NP DNA duplex is conformationally much more rigid than the A-form duplexes formed by DNA and RNA. What causes the limited conformational flexibility of the phosphoramidate duplex?

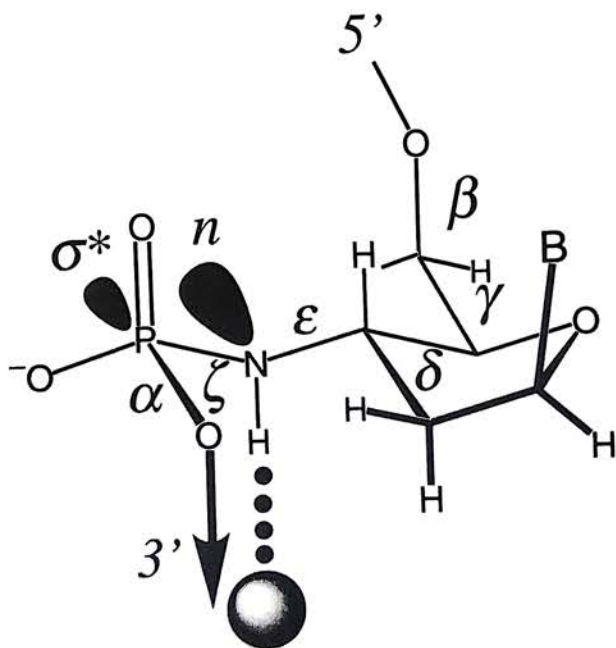




**FIG. 2.** Conformational rigidity of the backbone in 3'-NP DNA. The histograms depict the variations in the six backbone torsion angles  $\alpha$  to  $\zeta$  (in degrees) for the 72 nucleotides constituting the asymmetric unit in the 3'-NP DNA dodecamer crystals. The average values for backbone torsion angles are (standard deviations in parentheses):  $\alpha = -65.9$  degrees (10.7 degrees),  $\beta = 172.7$  degrees (10.7 degrees),  $\gamma = 54.4$  degrees (6.3 degrees),  $\delta = 79.3$  degrees (3.8 degrees),  $\epsilon = -153.4$  degrees (7.8 degrees), and  $\zeta = -72.6$  degrees (8.0 degrees) (Tereshko et al., 1998).

Although the crystal structure had established the overall conformation of the 3'-NP DNA duplex, its resolution (2 Å) would normally not have allowed a determination of the absolute configuration of the 3'-nitrogen. However, a direct hydrogen bonding interaction between the backbone amino groups and chloride ions at certain locations in the crystal lattice made it possible to distinguish between lone pair and hy-

drogen of the 3'-amino group (Tereshko et al., 1998). Accordingly, the lone pair of the 3'-amino nitrogen is positioned between the two nonbridging phosphate oxygens of the phosphate group. Thus, lone pair and polar P-O5' bond assume an antiperiplanar orientation, consistent with the anomeric effect (Fig. 3). Its role as a donor in hydrogen bonds to either chloride anions or water molecules in the crystal renders the amino ni-



**FIG. 3.** The antiperiplanar arrangement between the N3' lone pair  $n$  and the antibonding  $\sigma^*$  orbital of the P-O5' bond in the phosphoramidate moiety. The amino hydrogen is hydrogen bonded (dotted line) to either water molecules or chloride anions (gray sphere) in the crystal of the 3'-NP DNA dodecamer. This model is consistent with a destabilizing effect caused by bulky 2'-substituents, as this will create a 1-5 repulsion between the 2'-substituent and the acceptor hydrogen bonded to N3'. Thus, 3'-NP DNA strands containing a single 3'-amino-2'-methoxy substitution form significantly less stable duplexes with RNA and DNA strands than all-2'-deoxy-N3'  $\rightarrow$  P5' phosphoramidates (S. Gryaznov, unpublished observations). Their 2'-methoxy phosphodiester counterparts form duplexes with RNA that normally surpass the stability of the corresponding DNA-RNA hybrids by around 1°C per modification. However, small polar substituents at the 2'-position are tolerated, and oligo-2'-fluoro-2'-deoxy N3'  $\rightarrow$  P5' phosphoramidates paired to RNA display melting temperatures that are increased by 4°C–5°C per modification relative to the corresponding DNA-RNA hybrid duplex (Schultz and Gryaznov, 1996). Replacing O5' with an amino group will generate steric conflicts, in addition to sequestering the amino hydrogen from solvent access (Tereshko et al., 1998).



trogen electron richer. This would strengthen the conjugative effect between the nitrogen lone pair and the antibonding  $\sigma^*$  orbital of the P-O5' bond and should in principle shift the conformational equilibrium of the sugar further toward north!

The conformation of the oligonucleotide backbone in A-DNA duplexes could be derived from a qualitative conformational analysis, suggesting that the phosphodiester conformation is dominated by the anomeric effect (Eschenmoser and Dobler, 1992). This analysis used the following criteria: (1) ideally staggered substituents around all single bonds, (2) avoid 1,5-repulsions between 1,3-substituents wherever constitutionally possible, and (3) the phosphodiester group adopts an *sc/sc* conformation, in accordance with the generalized anomeric effect. The only conformationally repetitive arrangement of a backbone that satisfies the above criteria is the (*-sc, ap, +sc, +sc, ap, -sc*) variant, present in A-DNA double helices. It is noteworthy that the third criterion, although chemically sound, remains a hypothesis. Although the conservation of an ( $\alpha$ -*sc, \gamma* +*sc*)-conformation in the phosphodiester moiety of DNA duplexes is consistent with the presence of an  $n$ - $\sigma^*$  conjugation, the precise energetic contribution of this conjugation to the duplex stability remains hard to assess.

The configuration of the 3'-amino nitrogen in the crystal structure attests to the important role of the anomeric effect in fixing the phosphodiester conformation. We can anticipate that the strength of the anomeric effect is increased as a consequence of replacing O3' with N3'(H). The strong conjugation between the nitrogen lone pair and the antibonding  $\sigma^*$  orbital of the P-O5' bond explains the reduced conformational flexibility of the backbone torsion angle  $\zeta$  (Fig. 3). However, it is not apparent why this should limit the  $\alpha$  torsion to the *-sc* range and prevent it from switching to the *ap* conformation. Nevertheless, the experimental data show that both  $\alpha$  and  $\zeta$  are strictly limited to the *sc* conformational ranges in the phosphoramidate DNA backbone. With  $\alpha$  fixed in the *-sc* range,  $\gamma$  cannot flip into the *ap* conformation either, as the adoption of the extended backbone variant mentioned above proceeds in a concerted way and involves a crankshaft-like motion around  $\beta$ . A further contribution to the rigidity of the backbone may stem from the presence of the amino hydrogen in 3'-NP DNA in place of a lone electron pair of O3'. This most likely provides additional conformational limitations for torsion angles  $\delta$  and  $\epsilon$ . The structural results are inconsistent with the thermodynamic parameters in the sense that the former indicate a higher conformational rigidity of the phosphoramidate DNA backbone. The large loss of entropy upon duplex formation is surprising, but another characteristic of 3'-NP DNA, namely, its extensive hydration, may play a role in the entropy change.

## HYDRATION

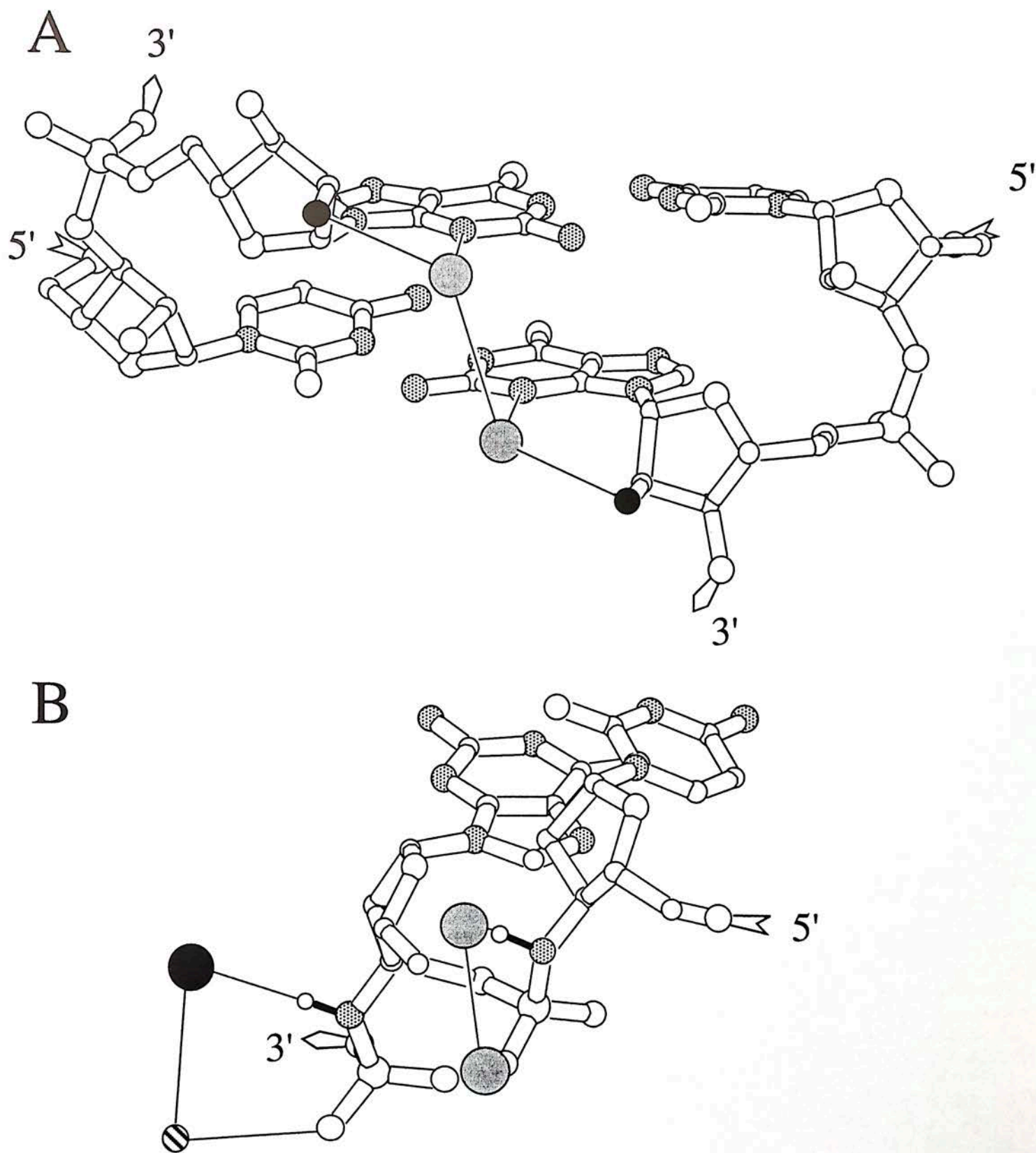
UV melting experiments have shown DNA duplex formation to be entropically favored relative to RNA duplex formation (for the 5'-CCCCGGGG model system, see Egli, 1996; Egli et al., 1996). However, the overall thermodynamic stability of RNA is higher, thanks to the large enthalpic contribution. The DNA duplex is conformationally flexible and can assume both, the A-form and the B-form. On the other hand, the conforma-

tion of double helical RNA is restricted to the A-form. One would assume an RNA single strand to be conformationally more restricted compared with DNA simply because the ribose is conformationally less flexible than the deoxyribose. Therefore, it is surprising that pairing of DNA strands results in a smaller loss of entropy than pairing of RNA strands. As in the case of 3'-NP DNA, the considerable loss of entropy upon duplex formation with RNA is counterintuitive and demands further examination.

A high-resolution crystal structure of an RNA duplex recently determined in my laboratory has revealed an extensive hydration of backbones and both minor and major groove (Egli et al., 1996). The RNA duplex features many more first-shell water molecules than the A-DNA duplex with identical sequence. The ribose 2'-hydroxyl groups line the border of the minor groove and are engaged in hydrogen bonds to three water molecules on average. Tandem water bridges are formed between 2'-hydroxyl groups of opposite strands across the minor groove (Fig. 4A). Thus, the introduction of a donor function into the backbone (the 2'-OH is also an excellent acceptor) leads to the immobilization of many more water molecules on the surface of the nucleic acid duplex. It is not unproblematic to compare molecular features at the atomic level to macroscopic properties, such as thermodynamic parameters. Although this is by no means the only interpretation, the structural results are in agreement with the following picture. The extensive hydration of RNA should bring about a strong enthalpic stabilization. Conversely, binding of a large number of water molecules on the RNA is expected to have a negative impact on the entropy. When we compare the stabilities of DNA and RNA, the contribution of hydration cannot be neglected. However, for the DNA and homo-DNA systems discussed earlier, the situation is somewhat different. There, one would expect the duplex hydration to change only marginally as a result of inserting a methylene group into the deoxyribose ring. Comparison of the thermodynamics of pairing for DNA and homo-DNA, therefore, offers a purer look at the effect of conformational preorganization of the single strand on duplex stability. The contribution of single-strand order to duplex stability can be measured, of course, but it is unclear if the contributions of conformational preorganization and hydration can be dissected.

Replacement of the DNA 3'-oxygen with an amino group in 3'-NP DNA also introduces a donor function into the backbone. Moreover, the 3'-amino group is a better acceptor than the bridging phosphate oxygen. Not surprisingly, hydration of the 3'-NP DNA duplex is dramatically improved relative to phosphodiester DNA (Tereshko et al., 1998). Not only is the phosphoramidate backbone extensively hydrated (Fig. 4B), but the presence of the amino groups also results in stabilization of water bridges across the minor groove (data not shown). It is likely that the excellent hydration of the backbone contributes to its rigidity. Taken together, these results suggest that the phosphoramidate DNA single strand is poised toward formation of an A-form duplex. Although this should result in entropic stabilization of duplex formation, it is feasible that the extensive hydration of the duplex compensates for this effect. The recent high-resolution crystal structures of double-helical RNA and 3'-NP DNA fragments support the view that water crucially affects duplex stability.





**FIG. 4.** (A) Hydration of RNA. Example of a tandem water bridge across the minor groove, with 2'-hydroxyl groups serving as bridge heads. 2'-Hydroxyl oxygen atoms are black, nitrogen atoms are stippled, water molecules are gray, and hydrogen bonds are included as thin solid lines. (B) Hydration of N3'  $\rightarrow$  P5' phosphoramidate DNA. Examples of tandem water bridges between 3'-amino nitrogen and phosphate oxygen. In some locations, a chloride anion and an ammonium cation replace the water molecules. Nitrogen atoms are stippled, the N3'-H3' bonds are filled, the chloride ion is black, the ammonium ion is hatched, water molecules are gray, and hydrogen bonds are included as thin solid lines.



## DISCUSSION

I have discussed the roles of single-strand order and hydration in nucleic acid pairing. The structural results suggest that both are of crucial importance for duplex stability. However, the individual contributions are difficult to assess. It should be helpful in this respect to conduct accurate thermal denaturation experiments with a variety of nucleic acid analogs. Understanding the origins of stability gains and correlating the thermodynamic and 3D structural data will provide the tools for a rational design of novel nucleic acid modifications with a host of desired properties.

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