Nitrogen-15-Labeled Oligodeoxynucleotides. 7. Use of ^{15}N NMR to Probe H-Bonding in an O^6MeG·C Base Pair

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The O^6MeG·C mispair is the most stable of the possible O^6-MeG·N mispairs, although the differences in stability are generally small.[1-4] Nevertheless, in spite of the slightly greater stability of the O^6MeG·C pair in a duplex, O^6MeG residues have been shown to direct preferential incorporation of thymidine upon replication.[5] Such a failure of a polymerase to follow the stability order seen in studies of duplexes, which has been observed also for 2-aminopurine residues,[6] may be understood based on a tight-binding model of the polymerase active site which excludes base pairs which differ significantly from Watson–Crick geometry.[3,4,6,7] Thus, while the O^6MeG·T pair is generally the least stable, its preferential incorporation may be the result of a more Watson–Crick-like geometry. However, the O^6MeG·C pair also can have a Watson–Crick-like geometry, particularly if the cytosine imino nitrogen is protonated. In fact, a ^{1}H NMR study in $CDCl_3$ using derivatized monomers found no evidence for base pair formation with the unprotonated cytosine derivative, while there was evidence for pairing with the O^6MeG derivative after protonation.[8] Moreover, a Watson–Crick-like O^6MeG·C orientation was found in an X-ray structure, albeit of a molecule with a Z-DNA conformation.[9] A ^{1}H and ^{31}P NMR study, at pH 7, of a molecule of the same sequence as that reported below had shown that the O^6MeG·C pairs were stacked in a right-handed DNA duplex.[10] Although there was no direct evidence for pairing found in that study, the wobble pair shown in Figure 1 was proposed tentatively. We now report that the O^6MeG·C pairs in this molecule exist in two distinct conformations, depending on pH, and that in each conformation there is H-bonding to both the O^6MeG N1 (acceptor) and N2 (donor) nitrogens. These results are most consistent with the two pairing schemes shown in Figure 1.

The [1-^{15}N]- and the [2-^{15}N]-O^6-methyl-2′-deoxyguanosines used in this work were obtained from the corresponding ^{15}N-labeled 2′-deoxyguanosines by conversion to the 6-O-pentafluorophenyl derivative followed by reaction with sodium methoxide.[11,12] The two ^{15}N-labeled molecules used for these ^{15}N NMR experiments, d[CGCGAATTC(^{15}N^{1}O^{6}Me)GCG] (**1**) and d[CGCGAATTC(^{15}N^{2}O^{6}Me)GCG] (**2**), are identical in sequence, differing only in the position of the ^{15}N label, and are self-complementary. Each was prepared by an H-phosphonate method on a 36-μmol scale.[13] The nitrogen chemical shifts reported were measured directly, at 40.5 MHz, on a sample consisting of a 1:1 mixture of **1** and **2**, at a total strand concentration of 11 mM.

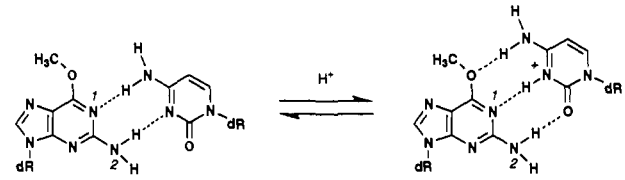

Figure 1. A rotamer of an O^6MeG·C wobble pair (left) and a resonance form of a protonated O^6MeG·C pair (right).

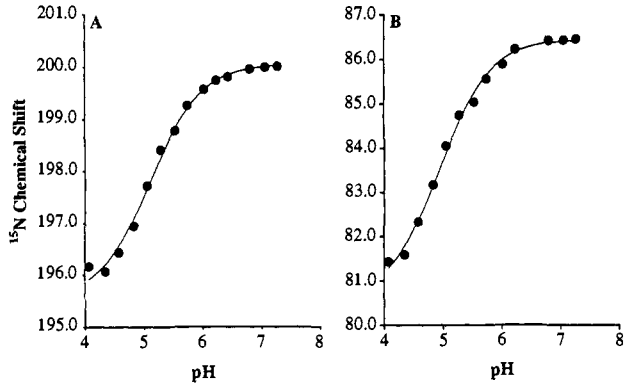

Figure 2. Plots of the O^6MeG ^{15}N1 (A) and ^{15}N2 (B) resonances as functions of pH. Chemical shifts are reported relative to NH_3, using external 1 M [^{15}N]HNO_3 in 90% D_2O at 25 °C at 375.8 ppm as a reference.[16] The sample consisted of a 1:1 mixture of **1** and **2** at a total strand concentration of 11 mM in 90% H_2O/10% D_2O, 0.1 M NaCl, 10 mM EDTA. The pH was adjusted by addition of NaOH or HCl. A nonlinear least-squares fit to the data gives the curve shown, from which pK values of 5.11 ± 0.04 (A) and 4.94 ± 0.04 (B) were obtained. The chemical shift data and relevant equations are included in the supplementary material.

The pH dependence of the chemical shifts of these O^6MeG ^{15}N1 and ^{15}N2 resonances is shown in parts A and B of Figure 2. Both the ^{15}N1 and ^{15}N2 resonances shift upfield as the pH is lowered from over 7 to near 4. For the ^{15}N1 the shift is 4.5 ppm *away* from the monomer or single-strand chemical shift, while for the ^{15}N2 it is 5.8 ppm *toward* the monomer or single-strand chemical shift. Although there is no low-pH base line in either case, a nonlinear least-squares[14] fit to the data gives pK values of 5.1 and 4.9 from **1** and **2**, respectively, for this transition. These apparent pK's, presumably for protonation of the cytosine imino nitrogen, correspond to only a modest increase from the 2′-deoxycytidine value of 4.3.[15]

The temperature dependence of the chemical shifts of these O^6MeG ^{15}N1 and ^{15}N2 resonances was monitored at both pH 7 and pH 5 and is shown in parts A and B of Figure 3. A nonlinear least-squares[17] fit to the data gives the melting temperatures and thermodynamic values listed in Table I. At pH 7 the ^{15}N1 resonance is shifted upfield by 3 ppm, and the ^{15}N2 resonance is shifted downfield by 8.3 ppm, relative to the corresponding single-strand values.[18] These are the directions of the chemical

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(18) These chemical shift changes are relative to a base line drawn by extrapolation of the high-temperature (single strand) chemical shifts to room temperature. The single strands would normally be hydrated, with the amino hydrogen atoms H-bonded to water oxygen atoms.

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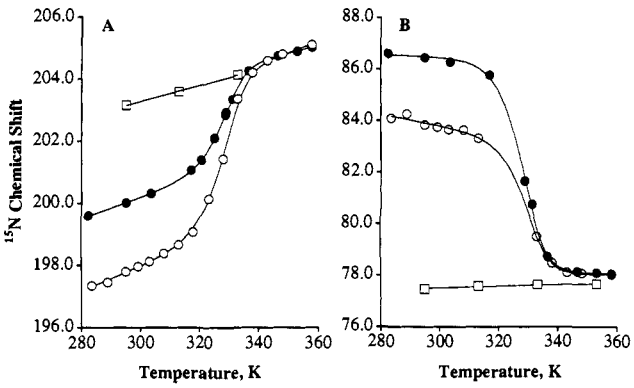

Figure 3. Plots of the O^{6}MeG ^{15}N1 (A) and ^{15}N2 (B) resonances as functions of temperature at pH 7 (●) and at pH 5 (○), and plots of [1-^{15}N]-d(O^{6}MeG) (A) and [2-^{15}N]-d(O^{6}MeG) (B) at pH 7 (□). A nonlinear least-squares fit to the data gives the curves shown, from which the thermodynamic values listed in Table I were obtained. The chemical shift data and relevant equations are included in the supplementary material.

Table 1. Thermodynamic Properties of {d[CGCGAATTC(O^{6}Me)GCG]}$_2$

source/pH	$-\Delta H°$ (kcal/mol)	$-T\Delta S°$ (kcal/mol at 298 K)	T_m[a] (°C)
NMR/**1**/pH 7	78 ± 5[b]	68 ± 5[b]	55
NMR/**2**/pH 7	77 ± 11[b]	67 ± 11[b]	55
NMR/**1**/pH 5	75 ± 10[b]	66 ± 9[b]	55
NMR/**2**/pH 5	77 ± 21[b]	67 ± 19[b]	56
UV/pH 7	81[c]	68[c]	65

[a] Calculated using the equation $T_m^{-1} = [(R \ln C_T)/\Delta H°] + (\Delta S°/\Delta H°)$ at 1.1×10^{-2} M. [b] Standard error. [c] Determined as reported previously in 1 M NaCl, estimated error ± 10%.[2]

shift changes expected for duplex hydrogen bonding.[19] At pH 5, which is the pK of the conformational transition at 23 °C, the duplex will have, on average, one protonated O^{6}MeG·C pair and one unprotonated O^{6}MeG·C pair. The chemical shift observed is an average of the two shifts. The thermodynamic values obtained at pH 7 agree well with those obtained from UV melting studies carried out previously.[2] Somewhat surprisingly, the thermodynamic values obtained at pH 5 are identical to those obtained at pH 7.

The results presented above demonstrate that the O^{6}MeG·C pair studied exists in both a protonated and an unprotonated form, with a pK of 5 for the transition between them. Further, in each form the ^{15}N1 chemical shift in the duplex is upfield of the single-strand or monomer chemical shift, while the ^{15}N2 chemical shift in the duplex is downfield of the single-strand or monomer chemical shift. The directions of the chemical shift changes observed, together with their sigmoidal temperature dependence, establish that there is O^{6}MeG·C hydrogen bonding at both the O^{6}MeG N1 and N2 positions in both the protonated and unprotonated forms of this mispair. The two structures shown in Figure 1 are consistent with these results.

In a protonated O^{6}MeG·C pair the cytosine N3 would be the expected site of protonation since it is the most basic site, with an intrinsic pK_a of 4.3.[15] This is significantly more basic than is the O^{6}MeG N1 (pK_a 2.3).[20] In addition, protonation of the O^{6}MeG ^{15}N1 would bring about a much larger change in chemical shift than that observed.[21] The 0.7-unit increase in the apparent pK_a is much smaller than the 3-unit increase seen with poly C,[22] although the stabilization provided by protonation of the O^6-MeG·C pair at pH 5 is sufficient to offset the otherwise destabilizing effect of this low pH on the duplex, so that the T_m and thermodynamic values are the same at pH 5 and 7. Optical melting experiments for an O^{6}MeG·C pair in a related molecule had also found similar T_m's at pH 5 and 7.[4]

The magnitude of the chemical shift changes due to base·base hydrogen bonding should be influenced by the particular H-bond donor and acceptor groups involved. In the results reported above, the upfield shift of the O^{6}MeG ^{15}N1 at pH 7 (3 ppm)[18] is similar to the upfield shift of the adenine N1 we found in an A·T pair (2.6 ppm), where in each case the labeled nitrogen is an sp^2 H-bond acceptor.[23] In contrast, the downfield shift of the O^{6}MeG ^{15}N2 (8.3 ppm) is much larger for this sp^3 H-bond donor to sp^2 nitrogen acceptor interaction than the downfield shifts of either the adenine ^{15}N6 in an A·T pair (1.2 ppm) or the O^{6}MeG ^{15}N2 in an O^6-MeG·T pair (1.1 ppm), both of which are NH_2–O interactions.[24] (There was no shift in the O^{6}MeG ^{15}N1 resonance in this O^6-MeG·T pair.[24]) Protonation of the O^{6}MeG·C base pair brings about a further 4.5 ppm upfield shift for the ^{15}N1 and a 5.8 ppm upfield shift for the ^{15}N2. The upfield shift of the ^{15}N2, which is now an NH_2–O interaction, bring this resonance to about 2.5 ppm downfield of that of the single-strand chemical shift, which is then more like the differences we found for the adenine ^{15}N6 and the O^{6}MeG ^{15}N2 when H-bonded to a carbonyl oxygen. Thus, it appears that the chemical shift change of an amino nitrogen for H-bonding with an imino nitrogen is much greater than that for H-bonding with a carbonyl oxygen, as would be expected.

These results further demonstrate the unique ability of ^{15}N labeling to define hydrogen bonding at specific nitrogens in synthetic DNA fragments. The sensitivity of the nitrogen chemical shift to the nature of the H-bond donor or acceptor should be increasingly useful as more examples become available.

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Supplementary Material Available: Tables of the ^{15}N chemical shifts of **1**, **2**, and [1-^{15}N]- and [2-^{15}N]-*O*^{6}-methyl-2′-deoxyguanosine and the equations used for curve fitting (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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