Characterization of Protonated Cytidine in Oligonucleotides by ^{15}N NMR Studies at Natural Abundance

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Understanding the nature of protonated cytidine (CH^+) in DNA is of great current interest due to the role it plays in the formation of DNA triple helices and other nonstandard structures. The nitrogen nucleus has been recognized as a good reporter on properties of CH^+. ^{15}N[2-4] and ^{14}N NMR[5] and NQR[6] have been used successfully to examine the effects of protonation of model compounds cytosine, cytidine, and cytidine monophosphate (CMP) in aqueous and nonaqueous solvents. In oligonucleotides, the requirement to resolve a number of signals in limited shift range makes it necessary to resort to ^{15}N NMR. Low receptivity and natural abundance of ^{15}N have led investigators to prepare isotopically enriched compounds, and the difficulties in obtaining these have impeded such studies of oligonucleotides. While biosynthetic labeling can be used to prepare appropriate RNAs for ^{15}N NMR (e.g., refs 7 and 8), for deoxyoligonucleotides expensive and laborious chemical synthesis is needed for appropriate labeled samples. Results have been obtained, nonetheless, from several oligomers after such efforts (e.g., refs 9-11). With the enhancement of proton-detected heteronuclear experiments, ^{15}N NMR of the imino nitrogens of bases in duplexes can be examined without enrichment and under the kind of conditions typically used for ^1H NMR of DNA, and ^{15}N shifts are informative regarding interactions of the bases.[12] Here we apply this approach to provide novel information on the properties of CH^+ in two oligonucleotide systems. Analysis of ^{15}N shifts and $^1J_{NH}$ couplings obtained in this way reveal that the character of CH^+ varies with environment.

The tetranucleotide d(TCGA) at pH 4.51 and a mixture of the oligonucleotides $d(G(AG)_8)$ and $d(C(TC)_8)$ in a ratio of 1:2 at pH 5.04 have been examined. The arrangement for self-association of the first sequence in solution is still under study. The second sequence has characteristics of a triple helix,[13] with a sequence similar to some recently analyzed by ^1H NMR.[14,15] While ^{15}N shifts of the G and the T residues are respectively rather similar (Figure 1, Table I), the peak corresponding to the CH^+ changes substantially in its ^{15}N position between the two cases. The proton shifts for the CH^+, by contrast, show little variation. The $^1J_{NH}$

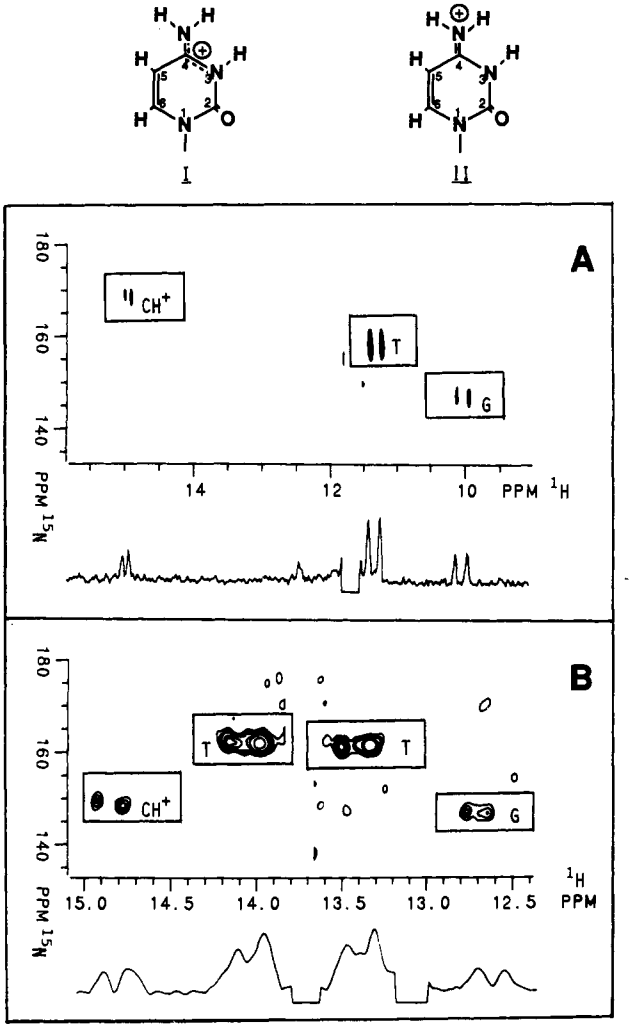

Figure 1. Contour plots of 2D proton detected ^1H-^{15}N HMQC spectra[23] of the imino region of (A) a solution of 160 ODU (optical density units) of d(TCGA) in 0.4 mL of 95% H_2O/5% D_2O (~10 mM in equivalent sites) at pH 4.51, 1 mM EDTA and 50 mM phosphate at 5 °C, along with ^1H projection, and (B) a solution of 322 ODU of $d(G(AG)_8)$ and $d(C(TC)_8)$ in a ratio of 1:2 in 0.4 mL of 95% H_2O/5% D_2O (~13 mM in equivalent sites) at pH 5.01, 0.1 mM EDTA and 10 mM phosphate at 25 °C. Signals in ^1H dimension appear as two peaks from $^1J_{NH}$ coupling. Regions in the proton dimension where artifacts from the transmitter and residual folded H_2O signals appeared were zeroed. The full proton spectral width, acquired in 1024 points for A and 512 points for B, is shown. For the ^{15}N dimension, 32 points were taken for a sweep width of 5000 Hz in both cases and zero-filled to 512 points for processing. Spectra are presented in the absolute value mode. Spectra were obtained and processed on a GN-500 spectrometer. Total experiment time in each case was about 21 h. ^1H and ^{15}N shifts were calibrated as previously reported.[24] To minimize water excitation, reduced proton excitation power was employed such that the inverse of the 90° pulse length was equal to the frequency offset of the transmitter from H_2O.[25] A composite ^1H 180° pulse was used in the middle of the evolution period providing an excitation profile similar to that of the 90° pulse without having to change power levels.[26] The 180° pulse is needed to generate a direct chemical shift correlation.[23] The ^1H frequency was set in the middle of the imino proton region. The ^{15}N excitation was centered at about 140 ppm from $^{15}NH_3$ with a 90° pulse width of 70 μs. Structure I at the top of the figure corresponds to CH^+ in TCGA, and structure II corresponds to that for the triple-helix system on the basis of analysis of the ^{15}N data.

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Table I. ^{15}N and ^{1}H Shifts and $^{1}J_{NH}$ Couplings of Imino and Imino-like Sites[a]

d(TCGA)				d(G(AG)$_8$) + d(C(TC)$_8$), 1:2			
base	N	H	$^{1}J_{NH}$	base	N	H	$^{1}J_{HN}$
G	147.2	10.14	-90	G	147.1	12.63	-74
T	158.5	11.44	-88	T	161.6	13.41	-85
				T	161.8	14.05	-85
CH$^+$	168.5	15.09	-47	CH$^+$	149.2	14.82	-74

[a] Couplings are in hertz and are assumed to be negative, ^{15}N shifts are relative to NH_3, and ^{1}H shifts are relative to (trimethylsilyl)-propionic acid, TSP. Assignments are based on ^{1}H NOESY results.

(Table I) also shows a substantial variation between the two systems examined, being an unusually low -47 Hz for the d-(TCGA) and -74 Hz for the triple-helix system. Typically $^{1}J_{NH}$ couplings of about -90 Hz[12,16,17] are found for imino nitrogens.

Shifts and couplings can be interpreted in terms of understood effects on ^{15}N.[2-4,17-20] Protonation of a nitrogen nucleus generally causes a large upfield shift. The magnitude of the coupling constant is indicative of the s character in the NH bond.[16] For CH$^+$ in DNA, direct comparison of shifts can be made with reported values for CMP in aqueous solution as a function of pH.[4] The N3 of CMP was found to move upfield 60 ppm from about 202 ppm to 144 ppm relative to NH_3 on protonation. Effects on couplings and shifts of modulating the NH bond have been studied in the context of azo-hydrazone tautomerism,[19,20] and both parameters were shown to reflect the degree to which the proton is associated with the nitrogen. Since the NH couplings could be observed in that case, as they can in the case of CH$^+$ reported here, the exchange regime for the ^{15}N resonances must be slow. Thus the shifts reflect the intrinsic NH bond interaction and are not just a fast weighted average of the limiting shifts.

In the triple helix, the shift of 147.1 for N3 of the CH$^+$ residue resembles the model value of 144 for protonated CMP.[4] This shift approaches the value for the *N*-methoxycytosine where N3 is protonated.[21] The $^{1}J_{NH}$ coupling seen in the triplex is comparable to the value for the other normal imino protons in the complex. (The width of the proton lines and the presentation in absolute value mode may introduce some inaccuracy in measurement of the splitting.) This leads to the conclusion that the N3 is essentially sp^3, leaving the bond between C4 and the exocyclic nitrogen largely double bond in character with the charge on this nitrogen (structure II). NQR data[6] on solid samples of cytosine salts suggests such an arrangement, as does ^{14}N NMR data.[5] Consistent with this analysis is the similarity of the shift between CH$^+$ and G imino ^{15}N resonances. The imino nitrogens of G have a carbonyl group as one neighbor and an sp^2 carbon as the other, while for this CH$^+$ the immediate neighbors are a carbonyl group and an sp^2 carbon with the double bond to the exocyclic nitrogen.

For the CH$^+$ in d(TCGA) the N3 resonance is about 20 ppm further downfield from the triplex, at 168.5 ppm, placing it almost exactly halfway between the positions of the protonated and nonprotonated N3 of aqueous CMP.[4] Further, the coupling is about half of the normal imino value. A weaker NH bond would imply less s bond character to N3 and would at least qualitatively explain both the intermediate coupling and shift for the d(TCGA) case, with analogy to the interpretation of the azo-hydrazone tautomeric system.[19,20] Thus, for the d(TCGA) system the positive charge of CH$^+$ would be delocalized between N3 and the amino nitrogen and their substituents (structure I), as was suggested for methylcytosine.[22]

It would have been informative to observe the amino nitrogens, but nonequivalence of these protons in the complexes and the greater proximity of these resonances to the H_2O resonance complicated matters, and we have not been able to observe them.

Although CH$^+$ bases can be observed by optical spectroscopic techniques,[27] ^{15}N NMR can provide more specific information about the characteristics of the base.

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Novel Method for NMR Spectral Correlation between the Native and the Denatured States of a Protein. Application to Ribonuclease A

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Recently, we have developed a new NMR technique, state-correlated two-dimensional (SC-2D) NMR spectroscopy, in which correlation of NMR spectra can be obtained between two chemically distinct states before and after a sudden temperature jump.[1,2] In the present communication, SC-2D NMR spectroscopy is applied for the first time to obtain a spectral correlation between the native conformer (N) and the denatured conformer (D) of a protein in solution.

Figure 1 shows the pulse sequence used to obtain the SC-2D NMR spectra of ribonuclease A (RNase A) in 0.15 M KCl, pH 1.0 in deuterium oxide. The pulse sequence resembles that of 2D exchange or 2D NOE, but the mixing period is replaced by the transition period during which a microwave pulse is applied for a temperature jump. The solution temperature increased from θ_1 = 30 °C to approximately θ_2 = 45 °C within 150 ms during or after which conformational transition (thermal denaturation) of the protein took place. Since the conformational transition can be complete during the transition period (200 ms) well within proton spin-lattice relaxation times of the protein (about 1 s at 400 MHz),[3] the spin magnetizations keep their amplitudes modulated by Larmor precession in the t_1 domain, even after they have been brought to the t_2 domain in which they precess with different precession frequencies, resulting in a 2D correlation spectrum after double Fourier transformation. The experiment was carried out at 400 MHz on a JEOL GX-400 NMR spectrometer with a homemade probe head.

Figure 2 shows the first successful observation of an SC-2D spectrum between N and D forms of RNase A in the aromatic proton region. Since at pH 1.0 RNase A transforms from an essentially N conformer to a fully D conformer by the temperature

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