A proton NMR study of a DNA dumb-bell structure with hairpin loops of only two nucleotides: d(CACGTG-TGTGCGTGCA)

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ABSTRACT

One- and two-dimensional nuclear magnetic resonance (NMR) experiments were used to study the conformation of the DNA hexadecanucleotide d(CACGTGTGTGCGTGCA) in aqueous solution. NMR spectra were recorded for the compound in D₂O and in H₂O/D₂O (90/10) over the temperature range 1°C–60°C. Assignments of imino proton resonances and of non-exchangeable proton resonances (except for some H4', H5' and H5'' resonances) are given. The ¹H-NMR spectra indicate that below about 20°C, the compound exists as a single monomolecular species. Between 20°C and 55°C the oligonucleotide occurs as a mixture of structures in fast exchange on the NMR time scale, except for the temperature region 30°–34°C, where substantial line broadening indicates intermediate exchange; above 60°C the single strand predominates. The imino proton spectra, chemical shift values, and scalar coupling and NOE data reveal that the monomeric form, which is exclusively present below 20°C, consists of a structure with a B-DNA double helix region of six base pairs, both ends of which are closed by hairpin loops of only two nucleotides, giving the molecule a dumbbell-like structure:

INTRODUCTION

Much of our knowledge about the conformational flexibility of DNA has been obtained by systematic investigation of synthetic DNA fragments in both the solid state and in solution (1). NMR spectroscopy has provided structures, thermodynamics and kinetics of DNA secondary structural elements (reviewed in reference 2). Dumbbell shaped molecules provide models not

only for hairpins, cruciforms and other DNA secondary structures with looped-out regions, but also for duplex DNA fragments containing a nick. Several NMR studies have been reported for hairpins (3–10), and a DNA minicircle formed by two sticky-ended hairpins has been studied (11). However, only studies of dumbbells employing ultraviolet spectroscopy, calorimetry and biochemical techniques have been reported (12–13); no detailed NMR study has been reported for a DNA dumbbell.

Here we present a conformational study of a DNA 16-mer which forms a dumbbell structure. The conformational characteristics of the loop regions of this dumbbell are compared with those of a closely related hairpin (6) and of other hairpin loops. These studies provide insight into the effect of loop sequence and size on loop conformation; the effect of a nick in a DNA duplex structure is also seen.

MATERIALS AND METHODS

The DNA oligonucleotide was synthesized on an Applied Biosystems 381A and purified by reverse phase HPLC; no phosphates are present on the 3' and 5' ends. After a threefold lyophilization from 99.96% D₂O, the sample was taken up in 0.8 ml 99.96% D₂O and the pH was adjusted to pH = 6.7 (meter reading). Half of this solution was lyophilized from 99.96% D₂O and dissolved in 0.4 ml 99.96% D₂O for observation of non-exchangeable proton resonances, while the other half was lyophilized and taken up in 0.4 ml H₂O/D₂O (90/10) for the detection of exchangeable proton resonances. The DNA concentration in the samples was 1.3 mM. TMA (tetramethyl ammonium ion) was used as an external reference. The TMA scale can be converted to the DSS (2,2-dimethyl-2-silylpentane-5-sulphonate ion) scale by the addition of 3.185 ppm at 4°C (14). We present all chemical shift data in terms of the DSS scale.

¹H-NMR spectra were recorded at 500 MHz on a General Electric GN-500 spectrometer. A series of spectra was recorded over the temperature range 1°C–60°C at five degrees intervals;

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spectra were recorded at two degrees intervals in the temperature range 22°C–42°C. Two-dimensional ^1H -NMR spectra were recorded at 4°C. Phase-sensitive NOESY spectra (mixing times 160 ms and 400 ms) were obtained using the TPPI method (15) with presaturation of the HDO peak. A total of 430 FIDs (each consisting of 120 scans) was collected and 2K datapoints recorded for each FID. Data were zero filled to 1K real points in t_1 and apodized prior to Fourier transformation using a skewed sine bell (phase shift 50°; skewness 0.7) in both dimensions. An absolute value COSY spectrum was recorded using a standard pulse sequence (15) with preirradiation of the HDO resonance. 256 t_1 values, with 128 scans per t_1 , were collected in 2K points. Data sets were Fourier transformed in t_2 dimension, zero filled and transformed in t_1 dimension yielding 1K by 1K data sets. Data were only apodized in t_1 by a 90° phase shifted sine bell. All 2D-data processing was done by means of the FTNMR program on a VAX 11/785 computer. NMR spectra of the compound in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10) were recorded between 1°C and 44°C, at five degrees intervals, using the 1-3-3-1 pulse sequence (16). 1D-NOE difference experiments were done at 4°C by means of the 1-3-3-1 solvent suppression pulse sequence in combination with an on/off presaturation pulse (0.4 s). 320 FID's collected with on-resonance presaturation were subtracted from 320 FID's with off-resonance presaturation; the resulting 1D-NOE difference FID was apodized, zero filled (8K-to-32K) and Fourier transformed.

RESULTS AND DISCUSSION

The spectral region of the thymine methyl resonances in the ^1H -NMR spectrum of the oligonucleotide in D_2O at 4°C is shown in Figure 1 (assignments are based on 2D-NOE experiments described below). Four distinct resonances of equal intensity are observed, corresponding to the four thymine residues in the hexadecanucleotide. Upon raising the temperature from 1°C to about 20°C, these resonances display a small downfield shift (0.025 ppm for Me(7) and Me(9) and 0.05 ppm for the Me(5) and Me(13). However, no significant line broadening, nor the

Fig. 1. The high-field region of the 500 MHz ^1H -NMR spectrum of $\text{d}(\text{CACGTGTGTGCGTGCA})$ in D_2O (1.3 mM DNA, pH 6.7, no added salt) showing the thymine methyl resonances at 4°C; peak marked X corresponds to an impurity. Thymine 7 and 9 have chemical shifts expected for B-form DNA, but thymine 5 and 13, with resonances near 1 ppm, are not in a B-form duplex conformation, nor in a single strand conformation.

appearance of a second set of methyl resonances at the expense of the original ones, was observed in this temperature range. Similar observations were made for the spectral region containing the base proton resonances. From these observations we conclude that the oligonucleotide exists as a single structure between 1°C and 20°C. Significant changes in the ^1H -NMR spectrum were seen upon increasing the salt concentration to 25 mM NaCl and the DNA concentration to 5 mM. Under these conditions, the methyl resonances of T(5) and T(13) shifted downfield from their unusual high field position at about 1.0 ppm (Figure 1). Now methyl peaks were only observed at positions in the spectrum corresponding to B-DNA-like structures (between 1.7 ppm and 1.3 ppm) (2). Thus with increasing salt and DNA concentration, dimers and possibly higher aggregates were formed. At 1.3 mM DNA and no added salt the data are consistent with a monomeric species.

Figure 2 shows the low-field region of the ^1H -NMR spectrum in $\text{H}_2\text{O}/\text{D}_2\text{O}$ at 4°C. Between 12 ppm and 14 ppm, the spectrum contains six sharp resonances (two for A·T base pairs and four for G·C base pairs); there is also a broad resonance at about 10.7 ppm. The appearance of this spectral region does not alter significantly in the temperature range 1°C–20°C except for additional broadening of the signal at 10.7 ppm. These findings indicate that the monomolecular species present in the sample between 1°C and 20°C consists of a double helical region containing two A·T and four G·C base pairs and has other exchangeable protons that are shielded from exchange with the solvent. Analysis of NOE, chemical shift and scalar-coupling data revealed that the six base pair double helical region has a B-DNA like structure, both ends of which are closed by loops consisting of only two nucleotides, giving the compound a structure which has been referred to as a dumbbell-like structure (13). Therefore, we will refer to the structure adopted by the deoxyribo-oligonucleotide between 1°C and 20°C as the 'dumbbell'.

Temperature-dependent behavior of the dumbbell structure

Figure 3 shows that the four methyl resonances display approximately a sigmoidal chemical shift vs. temperature profile. The plateau region below about 20°C is identified with the exclusive presence of the dumbbell-like structure. The small chemical shift changes observed in this region for Me(7) and Me(9) (1.25×10^{-3} ppm/°C) are associated with small and local conformational changes in the core of the duplex region. Similar observations for ^1H and ^{31}P resonances of nuclei located in the core of intact duplexes have been reported for duplex DNA oligonucleotides (2). The methyl resonances of T(5) and T(13) display a somewhat larger chemical shift change in this temperature interval (2.5×10^{-3} ppm/°C). Their behavior closely parallels that reported for the methyl resonance of the thymine residues located in the loop of the hairpin structure adopted by $\text{d}(\text{m}^5\text{CGm}^5\text{CGTGm}^5\text{CG})$ (5) and $\text{d}(\text{CCGAATT-CCG})$ (7), and indicates an enhanced conformational flexibility of the loop regions upon increasing temperature.

Significant changes in the chemical shift values of the methyl resonances are observed as the temperature is raised from 20°C to about 55°C. These chemical shift changes reflect the variation

in the relative fraction of structures (dumbbell, single strand and possibly other structures) undergoing exchange that is fast on the NMR time scale. Above about 55°C, the chemical shift profiles of the methyl resonances have a high-temperature plateau consistent with the predominant presence of single strands. The presence of a single structure below about 20°C (dumbbell) and above about 55°C (single strand) as deduced from the chemical shift vs. temperature profiles of the methyl resonances agrees with ultraviolet absorption vs. temperature curves for the oligonucleotide. These curves displayed a lower baseline up to 20°C and an upper baseline starting at about 60°C.

Fig. 2. The low-field region of the 500 MHz ^1H -NMR of d(CACGTGTGTGCGTGCA) in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10) (1.3 mM DNA, pH 6.7, no added salt) at 4°C. Six sharp imino proton peaks are seen as expected for the six base pairs; the four loop imino protons are in a broad lump near 11 ppm.

Fig. 3. Chemical shift versus temperature profiles of the methyl resonances in the spectra of d(CACGTGTGTGCGTGCA) in D_2O (1.3 mM DNA, no added salt). The sigmoidal curves imply a single species is present below 20°C that melts to a single strand by 50°C.

A significant line broadening (~ 50 Hz) was observed for all methyl resonances of d(CACGTGTGTGCGTGCA) between 30°C and 34°C, indicating intermediate exchange between the various structures. This observation contrasts with the temperature-dependent behavior of methyl resonances of thymines in DNA hairpin structures, e. g. those adopted by d(CCGAATTCGG)(7) and d(m⁵CGm⁵CGTGm⁵CG)(5). For these two compounds, engaged in a hairpin-single strand equilibrium, no significant line broadening was observed at any temperature. Thus, the dumbbell transition has slower kinetics than the corresponding hairpins. The slower kinetics of the dumbbell implies a higher activation energy for the dissociation of its double helical regions, each composed of three base pairs, relative to that for the dissociation of the hairpin structure, composed of a stem of three base pairs. Presumably the higher activation energy for the dumbbell-to-single strand transition relative to the hairpin-to-single strand transition is a result of the additional stacking interaction at the A(16)–C(1) and the T(9)–G(8) steps.

The chemical shift change experienced by the methyl resonances of the molecules during the dumbbell-to-single strand and the hairpin-to-single strand transition are about equal. The two hairpin stems of the dumbbell have similar stabilities as seen from the similarity of the chemical shift melting curves of Me(5) and Me(7) with Me(9) and Me(13). In addition, the transition midpoint of the dumbbell-to-single strand transition roughly coincides with that of the hairpin-to-single strand transition of d(CCGAATTCGG) ($\sim 35^\circ\text{C}$).

The low-field region of the spectrum of the compound in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10) at five temperatures is shown in Figure 4. As mentioned above, no significant changes are observed in this spectral region of the spectrum when the temperature is raised from 1°C to 20°C, except for a further broadening of the imino resonances of T(5), T(13), G(4) and G(12), located at about 10.7

Fig. 4. The low field region of the 500 MHz ^1H NMR spectrum of d(CACGTGTGTGCGTGCA) in $\text{H}_2\text{O}/\text{H}_2\text{O}$ (90/10) (1.3 mM DNA, pH 6.7, no added salt) at various temperatures. The temperature dependence of the resonances is consistent with a dumbbell structure. The imino peaks of the terminal base pairs (G8 and T9) do not show fraying; instead their behavior is similar to base pairs in the interior of a DNA duplex.

ppm, due to an enhanced exchange of these protons with the solvent. However, a line broadening is observed for all imino proton resonances when the temperature is increased from 19°C to 22°C. The resonances broaden further and the A·T signals decrease in intensity relative to the G·C resonances when the temperature is raised above 22°C. At 34°C, the A·T signals have broadened beyond detection and the G·C imino proton resonances are lost above 39°C. The resonances of the imino protons of the base pairs formed by the 3' and 5' terminal residues of the compound, i.e., C(1)·G(8) and A(16)·T(9) do not reflect fraying. This is different from the base pairs at the ends of DNA duplexes which exchange with solvent long before the interior base pairs do. Moreover, the broadening of the resonances of the imino protons of the base pairs adjoining the nick closely parallels that of the resonances of the imino protons in the core of the double helical regions. The stacking of the terminal base pairs of the dumbbell, and thus the exchange of their imino protons with solvent is similar to base pairs in a continuous double helix. Similar results are seen for the temperature-dependent behavior of imino proton resonances in the spectrum of a DNA minicircle (11).

Structural aspects of the dumbbell

The assignment of the imino proton resonances indicated in Figures 2 and 4 was done by means of 1D-NOE experiments on inter base-pair NOEs between the imino protons. The imino protons of the unpaired guanine and thymine bases in the loops,

G(4), T(5), G(12) and T(13), could not be assigned as they appear in the spectra only as a broad hump at about 10.7 ppm. Upon irradiation of the imino protons 1, 2, 15, 16, NOEs to their two neighboring imino protons were observed, while an NOE to only one imino proton was observed when the resonances 3 and 14 were presaturated. It is important to note that in addition to the expected NOE from the imino proton of base pair C(1)·G(8) to the imino proton of base pair A(2)·T(7), there was also an NOE of roughly equal intensity to the imino proton of base pair A(16)·T(9). This observation, in combination with the inter-nucleotide NOEs observed between the non-exchangeable protons of these residues, indicates an approximately normal stacking interaction between the base pairs adjoining the gap in one of the strands of the double helix.

Figures 5A and 5B show the parts of the NOESY spectrum containing crosspeaks between the base protons and the H1' protons, and between the base protons and the H2'/H2''/CH₃ protons, respectively. The base and H1' proton resonances were assigned using the standard sequential assignment procedure (17). The H2' and H2'' resonances were assigned from the cross peaks between the H1'-H2' and H1'-H2'' in the COSY spectrum; discrimination between the H2' and H2'' resonance of each residue is based on scalar coupling values (18) in combination with the difference in intensity of H1'-H2' and H1'-H2'' NOEs. The H3' resonances were assigned from intra-residue H1'-H3' NOEs in combination with intra-residue base-H3' NOEs. The H4' resonances of residues (4), (8) and (12) were easily identified

Fig. 5. Correlation area between the base protons and the H1'/H5 protons (A) and between the H8/H6 protons and the H2'/H2''/5 Me protons (B) in the NOESY spectrum of d(CACGTGTGCGTGCA) in D₂O at 4°C (1.3 mM DNA, pH 6.7, no added salt, mixing time = 160 ms). The duplex connectivity paths show that there is minimal distortion at the break between residues C1 and A16.

because of the isolated intra-residue cross peak between these protons and the H3' resonances. Using intra-residue H2'-H4' and H4'-H5' NOEs, the H4' and H5' resonances of residues (6) and (14) were assigned. The H4', H5' and H5'' resonances of residues (5) and (13) were assigned from the various crosspeaks between these resonances in the COSY spectra as well as from NOE cross peaks. The assignment of all these resonances is listed in Table 1 and was corroborated by the remaining cross peaks in the NOESY spectrum.

The NOE pattern observed in Figure 5A,B between base protons H6 or H8 and intra- and inter-nucleotide sugar protons H1', H2' and H2'' indicates that the double helical region of the dumbbell,

has a B-DNA like structure. The inter-residue NOEs observed between H6 of C(1) and the H1', H2' and H2'' protons of A(16), and between H6 of T(9) and the H1', H2' and H2'' protons of G(8) exclude the possibility that the double helical structure around the nick is significantly different from B-DNA even though the residues A(16) and C(1) are not covalently linked. Similar results have been reported for two DNA duplexes with nicks in the center (19). By contrast, for the dumbbell structure containing two loops of four thymine residues each adopted by a DNA 24-mer, it was proposed that the 5' and 3' ends at the nick are twisted relative to the helix axis (13). In a study of an analogous DNA 26-mer, (12) which adopts a dumbbell with loops of five thymine residues, it was found that the additional residue in each hairpin loop may permit the terminal residues at the nick to assume a collinear conformation.

An NOE pattern similar to the one described above is also observed at the C(3)-G(4)-T(5) and C(11)-G(12)-T(13) steps. However, note the reduced intensity of the G(4) H8-T(5) CH₃ and G(12) H8-T(13) CH₃ cross peaks relative to the normal G(6) H8-T(7) CH₃ and G(8) H8-T(9) CH₃ cross peaks. These results indicate an approximately regular stacking interaction at the C(3)-G(4)-T(5) and the C(11)-G(12)-T(13) steps, i.e. between the two nucleotides in each loop of the dumbbell and the 5' strand

of each stem of each hairpin. In contrast, no inter-residue NOEs are observed between base and deoxyribose protons at the T(5)-G(6) and T(13)-G(14) steps (the absent NOE cross peaks are indicated by dotted lines in Figure 5A and B). The absence of these NOEs reveal a stacking discontinuity at these steps, i.e. the stacking interaction between the last nucleotide of the hairpin loop and the 3' strand of the stem is significantly disturbed relative to the base-base orientation in B-DNA like structures.

Most of the protons in the dumbbell have chemical shifts corresponding to those in regular B-DNA (Table 1), however a few are different. The H2'', H3', H4' and H5', H5'' resonances of residues T(5) and T(13), and to a lesser extent the H5 resonances of residues C(3) and C(11) display a peculiar upfield shift, whereas the H2'' resonances of residues G(6) and G(14) display a small downfield shift. The methyl resonances of residues T(5) and T(13) are observed at an unusual high field position (1.0 ppm). In addition, Table 1 reveals very similar chemical shift values for the resonances of corresponding protons of residues C(3) and C(11), G(4) and G(12), T(5) and T(13) as well as G(6) and G(14). These findings indicate that the structure of the two hairpin-loop regions of the dumbbell structure are very similar and deviate from a regular B-DNA like structure, in agreement with the NOE results discussed above. The small differences in the chemical shift value for corresponding protons in the two loop regions are probably a consequence of long-range shielding effect by their different next-nearest neighbor, i.e. A(2)·T(7) vs. G(10)·C(15), and/or of a very small difference in the conformation of the two loop regions.

The conformations around some torsion angles of the phosphodiester backbone of the loop regions of the dumbbell, i.e. C(3)-G(4)-T(5)-G(6) and C(11)-G(12)-T(13)-G(14), could be derived from scalar and through space proton-proton interaction. The pseudorotation phase angle determined from sums of proton-proton coupling constants (20) revealed that the sugar rings of all residues occur predominantly in the S-type, approximately C2'-endo conformation.

The dumbbell conformation found for the oligonucleotide has several interesting characteristics. Its hairpin loops have only two unbonded bases and the B-form stacking of the stem regions continues into the loop on the 5' side of each stem. The stacking across the nick in the double helix is very similar to normal B-form stacking in an unperturbed duplex. Hairpin loops of two nucleotides have now been seen for sequences of -C-(G-T)-G-(5, 6), which is the same as found here, and for -C-(T-T)-G-, -T-(T-T)-A- (10). However, interchanging the order of the end bases, or substituting A's for T's eliminated the two-nucleotide hairpin loops (10). Whether a loop of two nucleotides forms, or a loop of four unbonded bases occurs, clearly will depend crucially on the DNA sequence. A hairpin loop of only two unbonded base has also been found in RNA (21).

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Table 1. Chemical shift values for the assigned resonances in the spectrum of d(CACGTGTGTGCGTGCA) at 4°C. Values are given relative to DSS (ppm).

	H5/Me	H8/H6	H1'	H2'	H2''	H3'	H4'	H5',H5''
C(1)	5.26	7.57	5.41	1.83	2.30	4.44	—	—
A(2)		8.09	6.15	2.34	2.70	4.85	—	—
C(3)	4.86	7.18	5.88	2.03	2.46	4.75	—	—
G(4)		7.64	5.53	2.08	2.47	4.55	4.10	—
T(5)	0.94	7.03	5.96	1.89	2.01	4.47	2.57	3.11,3.78
G(6)		8.13	6.17	2.80	3.06	4.83	4.39	3.84
T(7)	1.38	7.31	5.96	2.31	2.61	4.88	—	—
G(8)		7.80	6.02	2.53	2.78	4.90	4.39	—
T(9)	1.25	7.11	5.81	1.86	2.29	4.83	—	—
G(10)		7.78	5.70	3.90	3.90	—	—	—
C(11)	4.96	7.28	6.00	2.08	2.46	4.74	—	—
G(12)		7.78	5.70	2.16	2.50	4.59	4.18	—
T(13)	1.01	7.11	5.88	1.88	1.99	4.47	2.78	3.25,3.81
G(14)		8.00	6.09	2.70	2.95	4.78	4.33	3.80
C(15)	5.33	7.36	5.48	1.97	2.30	4.74	—	—
A(16)		8.26	6.28	2.81	2.65	4.72	4.26	—

The dashes indicate the resonances could not be assigned unambiguously; unassigned H4', H5' and H5'' resonances occur between 3.9 ppm and 4.3 ppm in the spectrum.

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