^{31}P NMR of a Hairpin Structure Within a 19-Mer DNA

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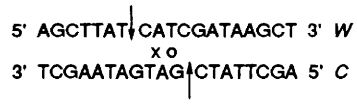
The ^{31}P resonances corresponding to the eighteen phosphate groups of a 19-mer DNA hairpin structure, belonging to a major cleavable site for topoisomerase II were unequivocally assigned and unusual ^{31}P chemical shifts corresponding to those phosphate groups located in the loop were noted.

There is a fundamental and pharmacological interest in learning how the spatial structure of the DNA cleavable sites of topoisomerase II is organized to permit a specific recognition by the enzyme. Topoisomerases II are enzymes which govern the topological state of DNA.[1] They are inhibited by antitumour agents which act in stabilizing the transient 'cleavable complex' consisting of the enzyme covalently attached to the 5′ ends of a 4 bp staggered DNA break.[2,3] These complexes occur at specific DNA sites, although the structural factors which govern selectivity are not well understood.

To identify unusual DNA structures as targets for topoisomerases II we started a NMR analysis of the strongest and salt-resistant cleavage site 22 in pBR322 DNA characterized in the presence of the antitumoral drug EPC (2,11-dimethyl-5-ethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazolium).[4] This site shows a remarkable twofold symmetry with a dyad axis (○) staggered relative to the symmetry centre of cleavage-site (×).



Owing to its inherent symmetry the oligonucleotide can adopt a hairpin structure, this being characterized by a stem stabilized by Watson–Crick pairs and ending with a three-base containing terminal loop (Fig.1). Such an idiosyncratic conformation of nucleic acids plays fundamental roles at the structural and functional levels.[5] Loop structures are of interest since they can allow specific tertiary interactions with other molecules including regulatory proteins.

This paper considers results obtained from a high-field ^{31}P NMR (202-MHz) analysis of the 19-mer DNA fragment found on the Crick stand (C) of the pBR322 cleavage site 22. The eighteen ^{31}P resonances of this heterogeneous oligonucleotide were assigned and particular conformational effects related to the hairpin structure identified. Experiments were performed at low ionic strength, thus favouring the monomolecular structure over the duplex structure. The assignment required the observation of the ^{31}P signals and their identification by heteronuclear chemical shift correlation spectroscopy. The simple and conventional 2D ^{31}P–^{1}H heteronuclear correlation (HETCOR) suffers from poor resolution in both the ^{1}H and the ^{31}P dimensions and has been used only in the past for oligonucleotides with up to five residues.[6] The ^{1}H–^{31}P reverse detection (^{1}H detection) experiment[7] has been developed as an alternative to assign the ^{31}P signals and applied to complex nucleic acid fragments.[8–11] On another hand the double constant time pure absorption phase heteronuclear correlation experiment, ^{31}P-^{1}H (DOC) was designed for phosphorus nuclei in large oligonucleotides. However, this technique has been used only scarcely and specta were always recorded at a low magnetic field (≈80 MHz).[6] Compared to the regular heteronuclear and COLOC experiments[6,12–14] the DOC pulse sequence has the capacity to reduce and practically eliminate the proton–proton coherence transfer that competes with the proton-heteronuclear coherence transfer. The DOC proved to be an appropriate procedure for our 19-mer DNA and was used throughout this work.

Strong arguments in favour of a hairpin structure are provided by NMR experiments in water. A number of NOESY connectivities from imino protons to amino and aromatic protons provided support for the postulated stem-loop secondary structure (Fig. 1). NOE effects concerned the imino protons of the five A–T (δ 13–14) and two G–C (δ 12.6–12.8) that are hydrogen bonded in the stem structure and their specific NOE patterns were identified. In contrast, the loop residues G_9, A_{10} and T_{11} did not present any NOEs while the presumed facing residues C_8 and G_{12} displaced only weak intensity NOEs, in accordance with their critical location at the junction of the mobile loop (results not shown).

Circular dichroism and UV-melting experiments also suggested the existence of a hairpin structure. UV experiments revealed single hyperchromic transition with melting temperature (T_m) = 63 °C at pH 7 and low ionic strength I = 0.1. The T_m for this transition was independent upon DNA concentration, indicative of an unimolecular process taking place at low salt concentration, therefore reinforcing the results obtained by NMR.

The 202-MHz 1-D phosphorus NMR spectrum of the oligonucleotide at 40 °C is shown in Fig. 2 together with the 1-D H$^{3'}$ and H$^{4'}$ NMR spectra and the corresponding 2D-DOC spectra. The chemical shift of each phosphate group is correlated to the chemical shift of those protons to which it is scalar-coupled.[5] In contrast to the reverse ^{1}H–^{31}P classical experiment the signals in DOC are decoupled in both dimensions. This feature, together with the good resolution in the two dimensions partly due to the high field used in these experiments, provides a clear identification of all the H$^{3'}$–^{31}P and H$^{4'}$–^{31}P cross-peaks in the spectra. This step is crucial for a safe assignment of the H$^{3'}$ and H$^{4'}$ protons, which was achieved by comparison of the NOESY and HOHAHA spectra after the sequential assignment of almost all the DNA protons, except H$^{5'}$H$^{5''}$ (severe overcrowding), was realized.[15–17]

The eighteen ^{31}P resonances arising from the eighteen phosphate groups in the 19-mer DNA occur between δ −4.0 and −4.4 from trimethylphosphate (TMP) (Fig. 1). The

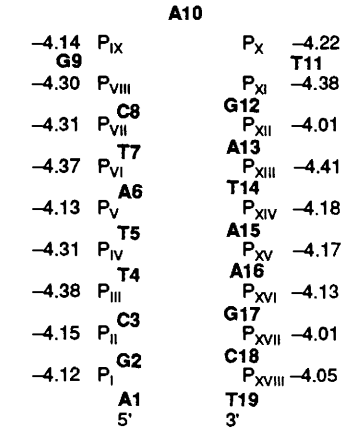


Fig. 1 The hairpin structure of the 19-mer DNA fragment. Bases and phosphate groups are numbered and ^{31}P-chemical shifts measured at 40 °C for an oligonucleotide solution in 10 mmol dm^{-3} phosphate buffer, 1 mmol dm^{-3} EDTA and 20 mmol dm^{-3} NaCl, pH = 6, are indicated (ppm from external trimethylphosphate).

phosphate groups in standard B-DNA[18] display chemical shifts clustered at δ −4.26.[19] A shift deviation from this average value can reflect rotamer population changes about the phosphodiester bonds (α, ζ and ε for example)[6,18] occasioning the local backbone distortions visualized both in solution[6,12–14] and in the solid state.[20] Most of the data presented confirm the chemical shift gradation previously reported for B-DNA phosphate groups by our laboratory *i.e.*: Pu–Py (AC, AT, GC, GT) ≥ Py–Py (TT, CT, TC) > Pu–Pu (AA, AG, GA) > Py–Pu (CA, CG, TA, TG).[12–14] However, there are several anomalous chemical shifts in addition to those corresponding to the end and penultimate steps submitted to fraying effects. The resonance of the loop $A_{10}pT_{11}$ step occurs at particularly low field (δ −4.22) relative to resonances of the stem A_6pT_7 (δ −4.37) and $A_{13}pT_{14}$ (δ −4.41) steps. Presumably, related to its particular conformation at the top of the loop, the ApT step exhibits an atypical chemical shift that becomes comparable to that of its reverse version TpA[21] within the stem. In the alternating poly-(dAdT)·poly(dAdT) B-DNA the ApT phosphate in the g-, g-(α, ζ) conformation is observed 0.2 ppm upfield to the phosphate TpA in the t, g- (α, ζ) conformation.[6] The other unusual feature concerns the two Py–Pu steps C_8pG_9 and $T_{11}pG_{12}$ which are located at the 5′- and 3′ sides of the loop, respectively. Resonances are strongly highfield shifted (roughly 0.4 ppm) from the common values in B-DNA suggesting that distortions occur in the phosphate backbone of these steps. Curiously, the G_9pA_{10} also located in the loop does not display an anomalous chemical shift.

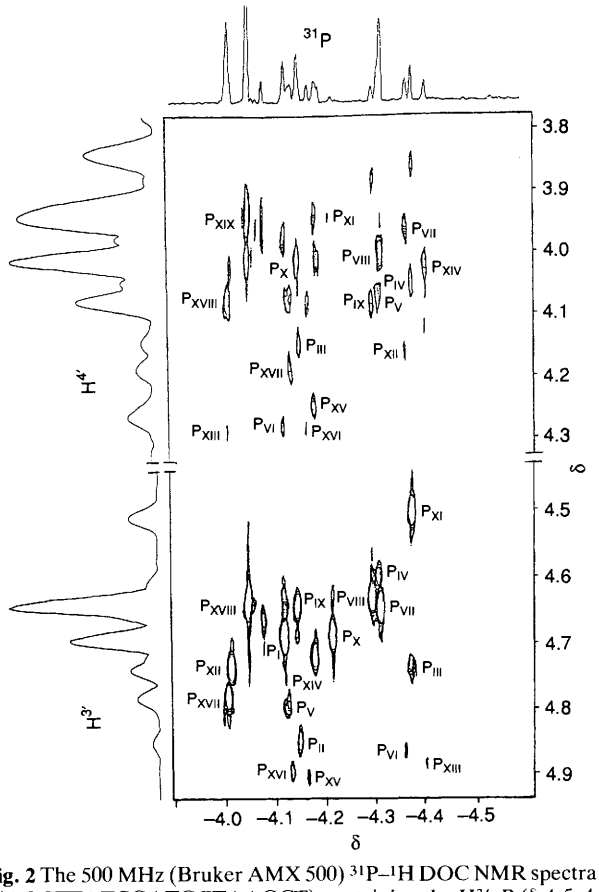


Fig. 2 The 500 MHz (Bruker AMX 500) ^{31}P–^{1}H DOC NMR spectra of d(AGCTTATCGATGCTAAGCT) containing the $H^{3'}$–P (δ 4.5–4.9) and $H^{4'}$–P, $H^{5'}$–P and $H^{5''}$–P (δ 3.8–4.3) at 40 °C. The $H^{3'}$–P and $H^{4'}$–P are labelled with roman numbers. The sample was prepared at a 3 mmol dm^{-3} DNA strand concentration in 10 mmol dm^{-3} phosphate buffer, 1 mmol dm^{-3} EDTA and 20 mmol dm^{-3} NaCl, pH 6. The two spectra were recorded separately. For the first spectrum ($H^{3'}$–P) the selective 180° pulse, characterized by DANTE loop consisting of 10 pulses each spaced by 150 μs, was centred on the $H^{3'}$ resonances and only were obtained the $H^{3'}$–P coherence transfer peaks. For the second spectrum ($H^{4'}$–P) the selective 180° pulse, characterized by DANTE loop consisting of 10 pulses each spaced by 150 μs, was centred on the $H^{4'}$ region. Both spectra were calibrated and the second spectrum was plotted on the first one. The data were collected with 2048 points in the ^{31}P dimension with 64 scans and zero-filled to 2048 × 512. The data sets were multiplied by a combination of an increasing gaussian function in the F2 (^{31}P) dimension and by a 45° shifted sine-bell function in the F1 (^{1}H) dimension before Fourier transformation in both dimensions. Each constant delay was 50 ms and the refocusing delay was 25 ms.

Thus, atypical chemical shifts are observed for several phosphate groups of our 19-mer heterogeneous DNA as a consequence of the implication of the corresponding steps within a DNA loop. The efficiency of ^{31}P NMR for detection of DNA backbone alterations and analysis of DNA polymorphism becomes then more evident. The results further point out the possible implication of atypical conformations such as hairpin or cruciform structures as recognition sites for nuclear enzymes such as topoisomerase II.

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