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^{31}P NMR investigation of the backbone conformation and dynamics of the hexamer duplex d(5′-GCATGC)$_2$ in its complex with the antibiotic nogalamycin

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Heteronuclear chemical shift correlation experiments confirm that the two down-field shifted ^{31}P resonances in the spectrum of the (nogalamycin)$_2$–d(GCATGC)$_2$ complex correspond to the phosphodiesters CpA and TpG at the intercalation sites. ^{31}P relaxation measurements (R_1, R_2 and {^{1}H}-^{31}P NOE) at 4.7 and 9.4 T permit the correlation time of each phosphate to be determined together with their chemical shift anisotropies. Significant differences in deoxyribose H3′–^{31}P coupling constants and chemical shift anisotropy contributions are observed, consistent with an asymmetric DNA backbone conformation for the phosphate groups at the intercalation sites. Large amplitude internal motions of the phosphates do not appear to contribute significantly to relaxation.

^{31}P NMR relaxation; (Nogalamycin)$_2$/d(GCATGC)$_2$; Chemical shift anisotropy; Correlation time; ^{1}H–^{31}P coupling

1. INTRODUCTION

Compounds that bind to DNA perturb its structure and function in such a way as to prevent its participation as a template in nucleic acid synthesis. Consequently, many potent inhibitors of transcription and replication have found use as clinical agents in the treatment of parasitic and malignant diseases [1,2]. Detailed structural studies of ligand–DNA complexes are important in understanding the physical basis for the biological activities of these compounds by defining not only possible recognition features but also the manner in which ligands perturb DNA structure, stability and dynamics.

The anthracycline antibiotic, nogalamycin (Fig. 1), is an intercalating agent that binds tightly to the DNA double helix. The interaction of the antibiotic with the hexamer duplex d(GCATGC)$_2$ has previously been described on the basis of solution NMR data [3], confirming that the molecule threads through the DNA helix positioning bulky sugar residues in both the major and minor groove simultaneously. In the complex, drug molecules are bound at each of the two equivalent 5′-CpA and 5′-TpG binding sites (represented schematically in Fig. 1) and appreciably stabilise the duplex to thermal denaturation. While many intermolecular NOEs define, with some precision, the position and orientation of the bound antibiotic, the effects of ligand binding on the phosphate backbone conformation and dynamics were not examined in detail. Large down-field perturbations to the chemical shifts of two ^{31}P resonances in the complex were, however, noted as diagnostic of an intercalative mode of binding. Since the negatively charged phosphodiester groups lie on the outside of the helix, and are thought to play an important role in protein–DNA recognition [4,5], the effects of ligand binding on their conformation and dynamics are further considered on the basis of ^{31}P NMR relaxation measurements and ^{1}H–^{31}P spin-coupling data.

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2. MATERIALS AND METHODS

The procedures used in the synthesis and purification of the oligomer, together with the formation of the 2:1 complex, have been described [3].

NMR spectra were recorded at 4.7 T on a Bruker WM 200 spectrometer, and at 9.4 T on a Bruker AM 400 spectrometer. Heteronuclear shift correlation experiments were performed at 4.7 T and 298 K using a spectral width of 500 Hz in F2 (^{31}P) and 800 Hz in F1 (^{1}H). Protons were decoupled using the Waltz 16 scheme. Phase-sensitive proton- coupled heteronuclear 2D spectra were acquired using the TPPI method [6]. 128 increments of 960 free induction decays, consisting of 1024 complex points were recorded, using a recycle delay of 2.5 s. Selective proton-decoupled ^{31}P NMR spectra were recorded at 9.4 T, with irradiation frequencies corresponding to the H3′, H4′ and H5′/5″ chemical shifts.

^{31}P spin-lattice relaxation rate constants were determined at 4.7 T and 9.4 T using the FIRFT [7] inversion recovery method employing 12 relaxation delays and a recyele time of 3 s. The data were analysed by non-linear regression to:

$$M(t) = a + b \exp(-R_1 t) \quad (1)$$

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where $M(t)$ is the magnetisation at time t, a and b are constants and R_1 is the spin-lattice relaxation rate constant. Spin–spin relaxation rate constants (R_2) were determined using FRESCO [8], the rapid spin-echo method, with 12 relaxation delays and non-linear regression to:

$$M(t) = M^0 \exp(-R_2 t) \quad (2)$$

where M^0 is the magnetisation at t=0. The {^{1}H}-^{31}P NOE was determined at 4.7 T using the gated decoupler experiment, with a relaxation delay of 10 s. The correlation times of the two cytosine H5-H6 vectors were calculated from time-dependent NOE measurements, as previously described [9].

3. RESULTS

3.1. *Resonance assignments*

Fig. 2 shows the broad-band proton-decoupled (A) and proton-coupled (B) ^{31}P NMR spectra of the (nogalamycin)$_2$–d(GCATGC)$_2$ complex at 4.7 T. The decoupled spectrum is similar to that reported previously, and highlights an unusually large chemical shift dispersion that is attributable to an intercalative mode of binding of the ligand [3]. The line-widths of the five resonances in the decoupled spectrum show little variation, indicating similar T_2 values for each phosphate. In the coupled spectrum the resonances are appreciably broader as a consequence of unresolved couplings to deoxyribose H3′, H4′ and H5′/5″. The apparent line-widths show significant variation as is particularly evident for the two resolved resonances at the low-field end of the spectrum. As the intrinsic line-widths are similar, the difference in apparent line-widths are attributable to variations in the magnitude of one or more ^{1}H–^{31}P coupling constants and indicative of differences in backbone torsion angles.

The heteronuclear shift correlation experiment has been used to assign the five phosphorus resonances (Fig. 3) with the aid of the proton assignments previously reported [3]. Each phosphate shows three proton correlations corresponding to the H3′ (4.7–5.2 ppm) of residue i, H4′ and H5′/5″ (3.8–4.6 ppm) of residue i+1. The relative intensities of the H3′-P cross-peaks vary substantially, again consistent with different ^{1}H–^{31}P coupling constants. The two lowest field resonances in the ^{31}P spectrum are readily assigned to the C2pA and T4pG phosphates at the intercalation site. The chemical shift values are presented in Table I.

3.2. *^{1}H–^{31}P coupling constants*

Figs. 2 and 3 indicate that there are significant differences in the ^{1}H–^{31}P coupling constants for the five phosphate groups. In the phase-sensitive coupled shift-correlation experiment (data not shown), C2pA and G1pC show a large anti-phase separation of about 8 Hz for coupling to H3′. Although this separation must be regarded as an overestimate of the true coupling constant, selective decoupling experiments show that the H3′-C2pA coupling is about 6 Hz, whereas all other H3′–^{31}P couplings are <3 Hz. According to Roongta et al. [10], the H3′–^{31}P coupling constant is related to the backbone torsion angle ε by the Karplus relationship:

$$^3J\text{H3′-P} = 15.3 \cos^2(\varepsilon + 120) - 6.1 \cos(-\varepsilon\text{-}120) + 1.6 \quad (3)$$

This equation has four solutions for ^3J < 10 Hz, two of which correspond to angles in the range $\varepsilon = -105°$ to $-180°$, which correspond to the B_{II} and B_I states of B-DNA, respectively, as found in the crystallographic data base. The data indicate that the C2p step is closer to the B_{II} conformation than the other residues.

3.3. *Relaxation rates*

A lower limit to the rotational correlation time of the complex is obtained from measurement of the re-orientational correlation time of the cytosine H5-H6

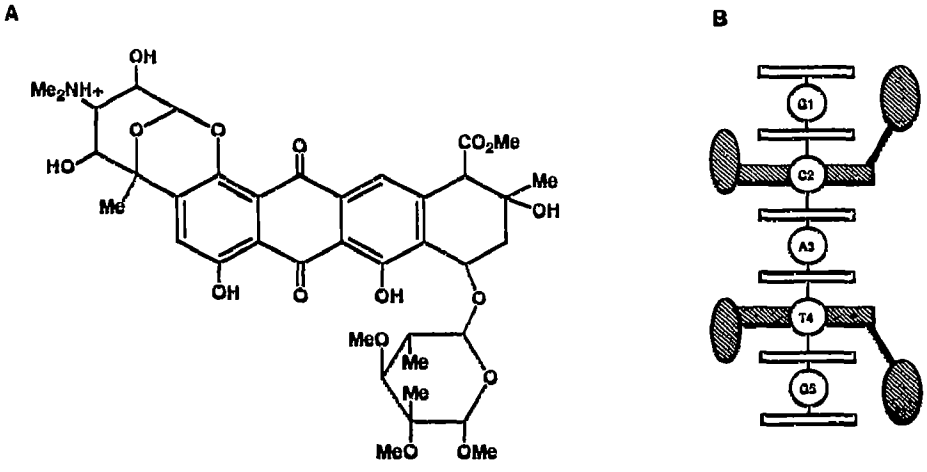

Fig. 1. (A) Chemical structure of nogalamycin, (B) schematic representation of the (nogalamycin)$_2$–d(GCATGC)$_2$ complex in which the two drug molecules are intercalated at the C2pA3 and T4pG5 steps. Circles represent phosphate groups labelled G1 through to G5.

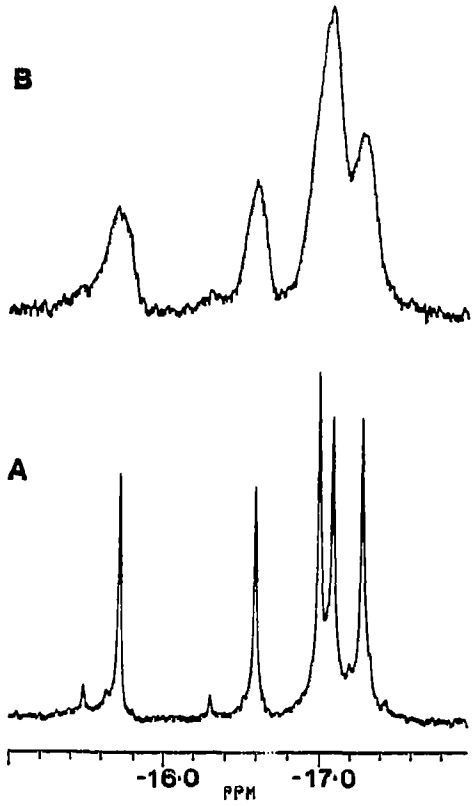

Fig. 2. ^{31}P NMR spectra recorded at 298 K and 81 MHz. Proton-decoupled (A), proton-coupled (B) 1D spectra.

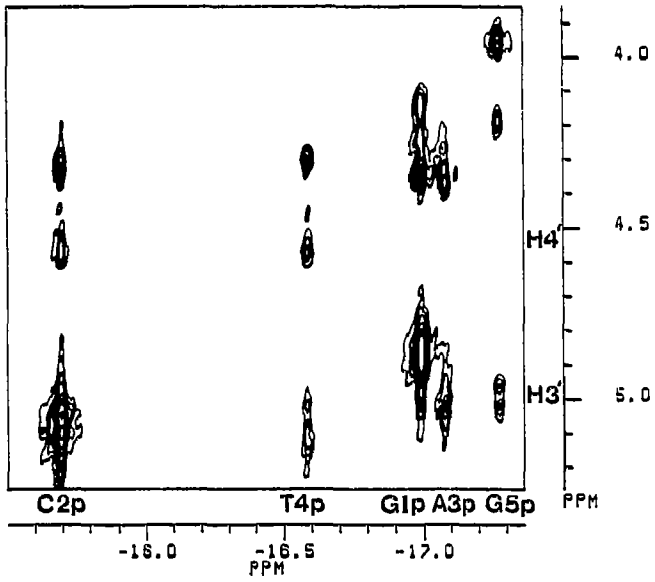

Fig. 3. 2D ^{31}P–^{1}H shift correlation spectrum recorded at 81 MHz and 298 K. 1024 data points were recorded in F2 (^{31}P) and 144 increments in F1 (^{1}H); the data matrix was zero-filled to 2048 by 1024 points prior to Fourier transformation using 60° shifted sine-squared apodisation function in both dimensions.

vectors. ^{1}H-^{1}H time-dependent NOEs were recorded on irradiation of the two cytosine H6 resonances (5 irradiation times), from which a cross-relaxation rate constant of $-0.56\ s^{-1}$ was obtained for both vectors at 298 K and 9.4 T. Assuming a fixed interproton distance of 2.46 Å, the correlation time is calculated to be 2.3 ± 0.3 ns. This value is 30% higher than that obtained at 298 K for the hexamer duplex d(CGTACG)$_2$ in a similar study [11]. With two drug molecules bound to the present hexamer the molecule is expected to be extended by nearly 7 Å (equivalent to an octamer duplex), and have a correlation time approximately 8/6 (i.e. 1.33) times as large as the hexamer alone [12], in agreement with experiment.

R_1 and R_2 values were determined at 4.7 T and 9.4 T, together with the {^{1}H}-^{31}P NOE at 4.7 T. The data are presented in Table I. Small differences in the relaxation rate constants among the five phosphates are apparent at the lower magnetic field strength. In all cases the NOE is small indicating the absence of large amplitude rapid fluctuations in the orientation of the P-H vectors that might affect relaxation. Indeed, the measured relaxation rate constants are very similar to those determined for a similar DNA hexamer [11]. The values of R_2 are small and are equivalent to intrinsic line-widths of about 1 Hz at 4.7 T. At a magnetic field strength of 9.4 T the R_2 values are approximately 2.5-fold larger, indicative of a much larger contribution to relaxation from chemical shift anisotropy (CSA). Differences in R_2 values among the five phosphates are more pronounced than at the lower field strength, the value of R_2 for the C2pA phosphate is now substantially smaller than for the others. As the contribution from dipolar relaxation is small at 9.4 T [11,13], an estimate of the effective correlation time τ can be obtained from the ratio R_2/R_1:

$$\tau = (1/\omega_p^2)\,[1.5\,(R_2/R_1 - 7/6)]^{1/2} \qquad (4)$$

The values determined, $\tau = 3.2 \pm 0.1$ ns, are very similar for all five phosphates, and are slightly larger than for the correlation time of the cytosine H5-H6 vector. This would seem to suggest that any substantial mobility of the phosphates on the subnanosecond time scale does not affect relaxation. Taking the value for the correlation time as 3.2 ns, the effective chemical shift anisotropy χ can be calculated for each phosphate from the data in Table I. The values range from 130 ppm for C2pA to 154 ppm for A3pT. It is apparent that the value of χ for C2pA is significantly smaller than the average value while that for A3pT is significantly larger.

4. DISCUSSION

The interaction of the anthracycline antibiotic, nogalamycin, with the hexamer duplex d(GCATGC)$_2$ has previously been shown to induce significant perturbations of base and sugar proton chemical shifts [3], alter

Table I

^{31}P NMR chemical shifts and relaxation parameters at 298 K[a]

Parameter	G1p	C2p	A3p	T4p	G5p
δ (ppm)[b]	−17.0	−15.7	17.1	−16.6	−17.3
4.7 T					
R_1 (s^{-1})	0.81	0.79	0.84	0.79	0.81
R_2 (s^{-1})	2.89	2.66	3.13	2.97	2.95
ΔR (s^{-1})	2.49	2.27	2.71	2.58	2.55
R_2/R_1	3.6	3.4	3.7	3.8	3.6
NOE	1.08	1.05	1.07	1.05	1.06
9.4 T					
R_1 (s^{-1})	0.84	0.80	0.95	0.86	0.92
R_2 (s^{-1})	7.2	6.2	8.3	7.1	7.4
ΔR (s^{-1})	6.8	5.8	7.8	6.7	7.0
R_2/R_1	8.6	7.8	8.7	8.3	8.0
τ (ns)	3.3	3.1	3.3	3.2	3.1
χ (ppm)[c]	142	130	154	141	144

[a] Errors in R_1, R_2 and NOE ± 10%; χ ± 5% and τ ± 10%.
[b] chemical shifts with respect to methylene diphosphate.
[c] Chemical shift anisotropy χ is defined by $\chi^2 = \Delta\sigma^2 (1 + \eta^2/3)$ where $\Delta\sigma$ and η are the anisotropy and asymmetry of the shielding tensor, respectively.

the position of the conformational equilibrium of the deoxyribose rings [14] and cause large down-field shifts of two of the five phosphate resonances [3]. The data are consistent with a significant drug-induced alteration of the average solution conformation of the hexamer duplex.

The down-field shifted phosphate resonances have now been assigned to C2pA and T4pG, corresponding to the sites of intercalation of the drug molecules in the complex. The differing extents to which the resonances of these two phosphates are down-field shifted (see Fig. 2), together with differences in H3′–P coupling constants and chemical shift anisotropies, provides a clear indication of asymmetric perturbations to the backbone conformation at the intercalation site. Recent crystallographic analysis of a nogalamycin–DNA complex [15,16] confirms that the basepairs forming the intercalation site are highly buckled and adopt an asymmetric wedge-shaped alignment.

The apparent correlation times describing the re-orientation of the C2 and C6 H5-H6 vectors are identical within experimental error, and are typical of those for nearly spherical molecules of this size isotropically tumbling in solution. While the apparent correlation times derived from the ^{31}P relaxation data are closely similar for all five phosphates, the mean value is 1.3 times larger than that obtained from the cytosine vectors. This difference has also been observed by NMR for other DNA sequences [11,17], and by comparison of the results of dynamic light scattering with NMR relaxation data [18], and may be a general observation. The correlation times calculated from the relaxation data must represent lower limits, as the effects of internal motion have not been considered. It is interesting that the phosphates, which are usually regarded to be the most mobile part of DNA duplexes, give correlation times larger than the cytosine bases, which are considered to be the most rigid part of the molecule [19,20]. If the chemical shift tensor is axially symmetric, and motions of the phosphate backbone occur about an axis parallel to the principle axis, then the relaxation rates will be insensitive to these motions. In this case, the apparent correlation time measured for the cytosine H5-H6 vectors will be approximately the 'true' correlation time scaled by the order parameter, which in the present case we calculate to be $S^2 = 0.75$ [21,22], and equate with librational motions of the bases within a cone of angle 25°, consistent with the findings of Eimer et al. [18]. Identical correlation times indicate that such motions appear to be very similar for the two cytosine bases despite the fact that one constitutes a terminal base pair, while the other interacts strongly with the aromatic rings of the antibiotic and has an unusually long basepair lifetime [3].

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