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^{31}P NMR study of daunorubicin-d(CGTACG) complex in solution

Evidence of the intercalation sites

E. Ragg, R. Mondelli, C. Battistini+, A. Garbesi* and F.P. Colonna*

Istituto di Biochimica e Chimica, Università di Milano, Via Celoria 2, 20133 Milano, +*Farmitalia Carlo Erba, Ricerca e Sviluppo, Via dei Gracchi 35, Milano* and **Istituto dei Composti del Carbonio contenenti eteroatomi e loro applicazioni, CNR, Ozzano Emilia, Bologna, Italy*

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The interaction of daunorubicin with the self-complementary DNA fragment d(CGTACG) was studied by ^{31}P NMR spectroscopy. The individual phosphates have been assigned for the nucleotide and the complex and signals from bound and free species in slow exchange at 19°C were detected. In solution, the hexanucleotide binds two molecules of daunorubicin, which intercalate in the d(CG) sequence at both ends of the helix. Evidence for local deformations of the backbone at the sites of C_5pG_6, C_1pG_2 and G_2pT_3 phosphates is given. The binding constants for the stepwise equilibrium and the rate of dissociation of the intercalated duplex were also determined.

^{31}P-NMR; Daunorubicin; DNA-drug interaction; Oligonucleotide

1. INTRODUCTION

The interaction of daunorubicin with DNA or low-M_r model systems has been extensively studied [1,2]. Several groups have attempted to evaluate the structural aspects of the daunorubicin-DNA complex on the basis of X-ray [1,3] and NMR analyses [4–7]. NMR studies performed on poly(dA-dT) and $d(TA)_3$ have suggested, on the basis of proton chemical shift values, that rings B and C of daunorubicin overlap with adjacent base pairs, while ring D extends out from the helix. The interaction with methylated deoxynucleotides containing only d(CG) sequences has been studied by ^{1}H NMR at low temperature [6]. This allowed evaluation of the binding constant for the complex between the tetramer d(CGm^{5}CG) and one molecule of daunorubicin, giving a value of 10^4 M^{-1}. Intercalation experiments with native DNA utilizing ^{31}P NMR have also been reported [7], suggesting a correlation between the ^{31}P chemical shift and unwinding angle. However, the local deformations induced in the deoxyribose phosphate backbone in solution have not previously been studied. The knowledge of detailed conformational changes at each phosphate site is important for characterization of the stereodynamic properties of DNA chains. In addition, the problem of base sequence selectivity of intercalation is still unsolved [2,8]. This aspect is of intense current interest, as it is essential to understand, on a molecular basis, the biological activity and to develop projects on more potent antitumor agents.

We report here results obtained from a study of the interaction of daunorubicin with d(CGTACG) in solution, by using ^{31}P NMR spectroscopy with a two-dimensional (2D) methodology, allowing the assignments of individual phosphate nuclei to be made. Phosphorus atoms are strategically located in the backbone of nucleotides, thus providing

Correspondence address: E. Ragg or R. Mondelli, Istituto di Biochimica e Chimica, Università di Milano, Via Celoria 2, 20133 Milano, Italy

* For the synthesis of the hexanucleotide

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unique conformational information on the phosphate chain, which is usually lacking in proton studies.

2. MATERIALS AND METHODS

Experiments were performed at 7.05 T over the temperature range 5–90°C, with a 10 mm broad-band probe and a 5 mm tube. Chemical shifts are given in ppm referenced from 1% H_3PO_4 in D_2O sealed in a capillary. 2D $^{31}P/^1H$ shift correlation spectra were recorded by using a modified HETCOR sequence. 2D exchange spectra were obtained by a phase-sensitive NOESY sequence with a mixing time of 0.05 s. 5 mg d(CGTACG) previously treated with Chelex-100 were dissolved in 0.5 ml of 99.96% D_2O containing 1 M NaCl, 1 mM EDTA and 10 mM phosphate buffer, pH* 6.7. The actual concentration was determined from the UV absorbance ($\epsilon^{260} = 53.63 \times 10^4\ M^{-1} \cdot cm^{-1}$) [9]. Variable amounts of a 63 mM solution of daunorubicin in D_2O were added to the hexanucleotide. d(CGTACG) Na salt was synthesized following the methodology of Van Boom et al. [10]. Nucleotides are numbered C_1–G_6 along each strand, going from the 5′- to 3′-direction.

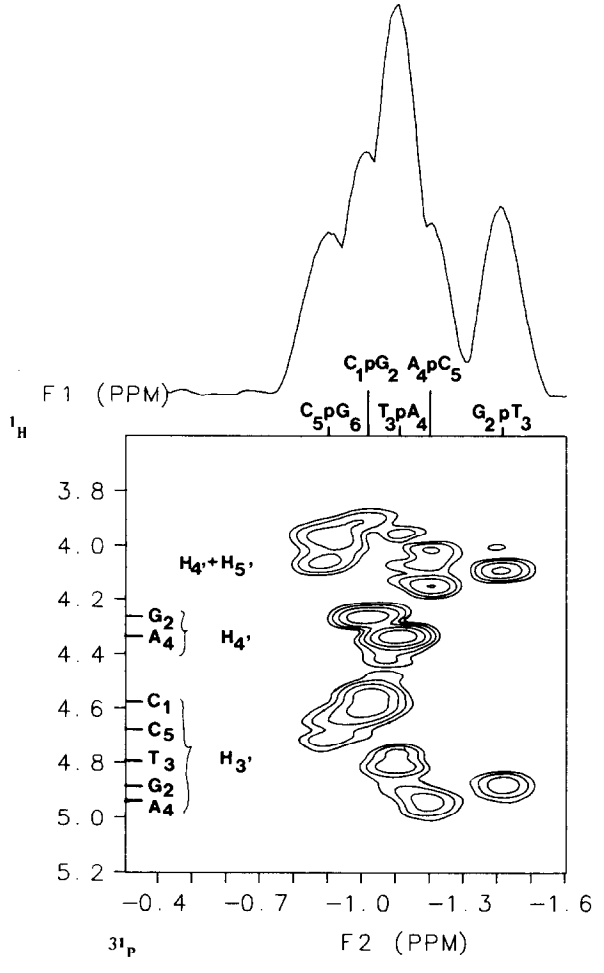

Fig.1. 2D $^{31}P/^1H$ shift correlation map of d(CGTACG) at 22°C. For this experiment a concentration of 10 mg/ml was used. The horizontal projection is reported on top.

3. RESULTS AND DISCUSSION

The assignments of ^{31}P nuclei in d(CGTACG) were performed by using the 2D $^{31}P/^1H$ shift correlation method. Three- and four-bond scalar couplings between ^{31}P nuclei in the phosphate backbone and the H-3′, H-5′ and downstream H-4′ deoxyribose protons are manifested as cross-peaks in the 2D spectrum of fig.1. Identification of these peaks requires knowledge of the proton assignments which have been performed via 2D NOESY and variable temperature experiments (to be published); our results are in agreement with those of Gronenborn et al. [11], who have shown, by ^1H NMR, that this nucleotide at 5°C in 1 M KCl exists in the B-DNA type conformation. As the three-bond coupling with C-5′ protons could not be utilized, their signals being undifferentiated, we focused on H-3′ and H-4′. The proton shift values are reported on the *y*-axis of fig.1 and identification of the cross-peaks is thus straightforward. Two of the H-4′ signals (A_4 and G_2) are sufficiently separated from H-5′ and their connectivities, through the four-bond couplings with T_3pA_4 and C_1pG_2 phosphates, confirm the assignments.

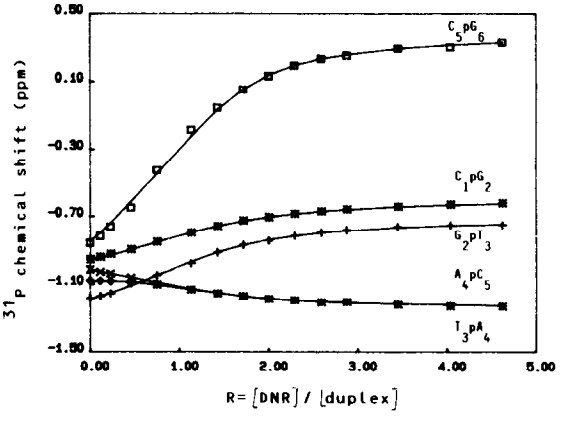

Fig.2. ^{31}P chemical shift variation as a function of the ratio R = [daunorubicin]/[duplex] at 45°C; DNR, daunorubicin.

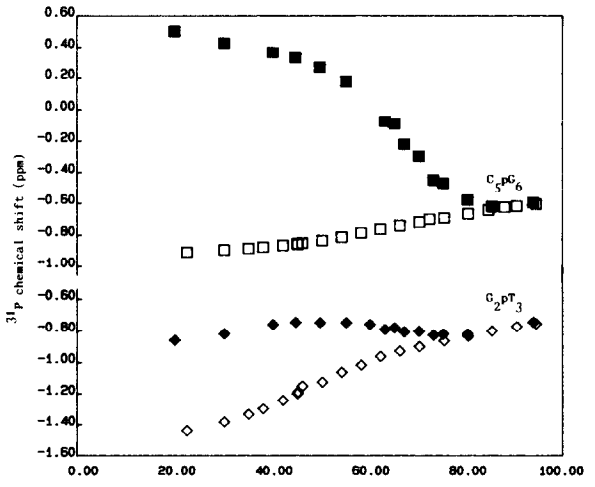

Fig.3. ^{31}P chemical shift variation of C_5pG_6 and G_2pT_3 signals for d(CGTACG) (□, ◇) and for the daunorubicin-d(CGTACG) complex (■, ◆) as a function of temperature.

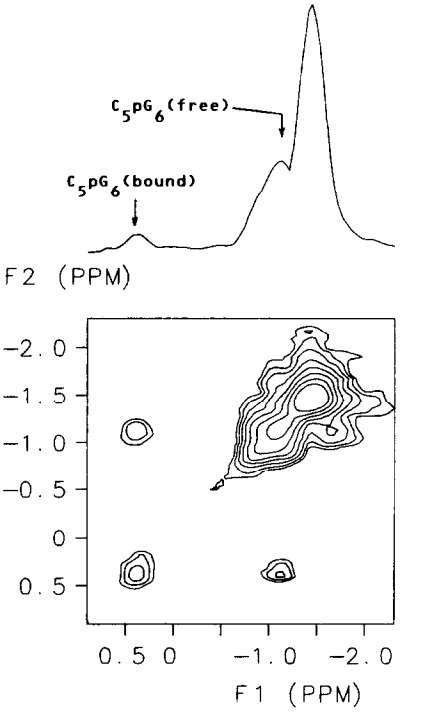

Fig.4. 2D ^{31}P NMR exchange spectrum of the complex daunorubicin-d(CGTACG) (R = 1) at 19°C. The 1D spectrum is reported at the top.

In the complex with daunorubicin, the assignment of phosphates was carried out by following each ^{31}P signal on addition of increasing amounts of daunorubicin, at 45°C (fig.2). At this temperature, the free hexanucleotide is 66% double helix; the equilibrium constant for the melting process has been derived from the curves of fig.3 (K_M = 1.5 × 10^{-3} M). However, the nucleotide, when bound to daunorubicin, exists entirely as the double helix even at 45°C, the intercalation acting as a stabilizing factor. At 45°C the signals are in the fast exchange regime, and are sufficiently narrow to be followed individually through the titration.

We calculated the association constants for the complex formation by considering the following equilibrium reactions:

$$\text{Duplex} \overset{K_M}{\rightleftharpoons} \text{single strand}$$

$$\text{Duplex} + \text{daunorubicin} \overset{K_1}{\rightleftharpoons} \text{duplex-daunorubicin}$$

$$\text{Duplex-daunorubicin} + \text{daunorubicin} \overset{K_2}{\rightleftharpoons} \text{duplex-(daunorubicin)}_2$$

$$2(\text{daunorubicin}) \overset{K_D}{\rightleftharpoons} (\text{daunorubicin})_2$$

The first equation describes the melting of the duplex, the final describing the aggregation of daunorubicin which resulted in being competitive with the intercalation process (K_D = 2 × 10^4 M^{-1} [12]). The calculated binding constants are K_1 = 2.0 ± 1.0 × 10^4 and K_2 = 1.4 ± 1.0 × 10^4 M^{-1}.

The chemical shift values of bound phosphates relative to the above equilibria have also been optimized by the fitting procedure; a remarkable downfield shift for C_5pG_6 (1.32 ppm) results on intercalation; lower shifts for C_1pG_2 (0.43 ppm) and for G_2pT_3 (0.53 ppm) are found, whereas A_4pC_5 and T_3pA_4 move slightly upfield of approx. 0.2 ppm. The strong and selective effect on C_5pG_6 indicates that the intercalation of the first and second daunorubicin occurs at the two symmetry-related C_5pG_6 sites. A similar lowfield shift has been observed in the complex actinomycin-d(AGCT)$_2$ [13].

The signal of C_5pG_6 is the only one which shows a remarkable temperature effect, as appears from the melting curves of fig.3. When the solution of molar ratio R = [daunorubicin]/[duplex] = 1 is

cooled below 30°C, the resonance of these phosphates splits into two signals. That the two signals belong to the free (−1.1 ppm) and intercalated (+0.4 ppm) species connected by slow chemical exchange follows from the NOESY experiment reported in fig.4. The dissociation rate constant calculated at the coalescence temperature (~30°C) is found to be 400 s^{-1}. The 1.5 ppm shift difference between bound and free C_5pG_6 phosphates is very close to the value measured (1.25 ppm) at the end of the titration experiments at 45°C, and to that obtained from the fitting procedure. The solution with R = 4.6 at 20°C shows only one signal for C_5pG_6 at +0.5 ppm. These results confirm the assignment of the intercalation sites.

The major contributing factors that determine ^{31}P chemical shifts in nucleotides are the chain torsional angles ζ = C(3′)-O(3′)-P-O(5′) and α = C(5′)-O(5′)-P-O(3′) of the phosphodiester groups along the double helix. The *gauche-gauche* conformation relative to these angles is favoured in the B-DNA family and is responsible for a more upfield shift, while the *gauche-trans* conformation is generally associated with a lowfield effect of approx. 1.5 ppm [13]. Consequently, we can say that daunorubicin induces an increase in the *g,t* conformation for C_5pG_6, C_1pG_2 and G_2pT_3. The significantly large lowfield shift observed for C_5pG_6 with respect to the others indicates strong helical deformation at the level of C_5G_6 sequences, as a consequence of the interaction. The much lower shift (0.3 ppm) of the partner phosphates C_1pG_2 is surprising, but must be associated with the asymmetric shape of the daunorubicin molecule (^1H NMR study of the complex is in progress). Actually, the X-ray structure of the daunorubicin-d(CGTACG) complex [3] shows a strong deformation for the phosphodiester angle ζ of C_5pG_6 (171.5°), while the same angle for C_1pG_2 retains the normal *gauche* value (67.5°). The lowfield shift of only 0.45 ppm obtained for G_2pT_3 seems to indicate small conformational changes at the level of these phosphate bonds, whereas in the crystal phase a ζ angle of 177.0° was measured, corresponding to a 'pure' *trans* conformation. The upfield shifts observed for A_4pC_5 and T_3pA_4 in the complex at 45°C are small but significant, as they show a return to the values of the free nucleotide at 20°C, thus verifying the increase in double helix population, at these sites, induced by intercalation.

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