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Direct observation of (H8,H6)–H1′ *J*-coupling correlations in oligonucleotides for unambiguous resonance assignments

Use of *J*-scaling in two-dimensional correlated spectroscopy

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Four-bond H8/H6–H1′ scalar coupling correlations in two-dimensional correlated spectroscopy have been observed directly for the first time by using the *J*-scaled COSY [(1985) Chem. Phys. Lett. 116, 105–108] technique in a dinucleotide, cytidylyl(2′–5′)guanosine (CpG). Unambiguous resonance assignment of non-exchangeable protons in CpG has been obtained using these H8/H6–H1′ 4-bond correlations and the various 3- and 4-bond sugar ring proton correlations observed in the COSY and SUPER COSY experiments.

J-scaled COSY *2D NMR* *Oligonucleotide*

1. INTRODUCTION

Two-dimensional (2D) NMR has found extensive use in resonance assignments and structure determination of oligonucleotides [1–10]. 2D homonuclear correlated spectroscopy (COSY) has been used to identify the sugar ring ^{1}H resonance positions by following their connectivity networks. It also helps to identify the resonances of thymine and cytosine base protons. The COSY spectrum, however, does not enable assignment of these spin systems to particular nucleotides along the sequence of the molecule. This problem has been circumvented in the past by the use of 2D nuclear Overhauser enhancement spectroscopy (NOESY) in moderately large oligonucleotides in which correlations between base protons and sugar protons can be observed. Since the NOESY spectrum reflects dipolar coupling correlations, cross-peaks identify hydrogens which are close by in space. Accordingly different strategies have been evolved for the interpretation of NOESY spectra in terms of known DNA structures. These are then used to obtain resonance assignments for the individual hydrogens in a sequential manner jumping from one nucleotide to the adjacent one. Consistency of the calculated NOESY spectra, on the basis of the known different DNA structures, with the experimental NOESY spectrum helps in identification of the structure of the nucleotide under study. Two types of difficulties are often encountered in such a procedure; (i) internucleotide NOEs are not observed due to lack of rigid structures, which is a common feature of small molecules and (ii) overlap of chemical shifts in larger molecules renders difficult, unambiguous assignments of cross-peaks in the NOESY spectrum. With respect to H1′ protons, this problem is schematically depicted in fig.1. In B-DNA structures, each proton is expected to show NOESY cross-peaks to 2 H1′ protons, one belonging to the same nucleotide, and the other to the previous nucleotide in the sequence (the numbers increase from the 5′-end to the 3′-end). Assignment of these peaks will be unambiguous if their positions are as shown in fig.1a. On the other hand, if the situation is as shown in fig.1b or c, it is clear that unambiguous assignment is not possible. Further-

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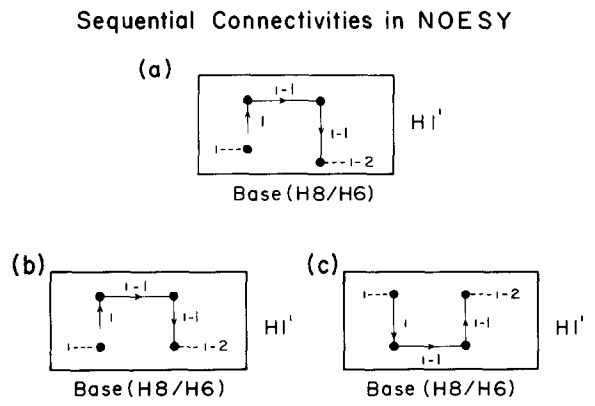

Fig.1. Schematic representation of sequential assignment of base H8/H6 and sugar H1′ protons in NOESY. The chemical shifts of H1′ protons are represented along the vertical direction and those of the base protons along the horizontal direction. The label on the vertical lines indicates the base H8/H6 assignment and the label on the horizontal lines indicates the sugar H1′ assignment. In (a) no overlap between H1′ protons leads to unambiguous assignment of base H8/H6 protons and sugar H1′ protons. In (b) and (c) overlap of sugar H1′ of i and $i-2$ residues leads to ambiguous assignment of the H1′protons.

more, in practice there can be situations where only one cross-peak is observed from the base proton, either because the structure is different or because of certain other factors, and then it is not possible to say, a priori, whether this corresponds to the H1′ proton of the same nucleotide or to that of the previous nucleotide. It would therefore be of great help if base-H1′ correlations of individual nucleotides could be established independently by other means. These coupling constants have been measured by one-dimensional (1D) techniques in mononucleotides, and have been found to be ~0.3 Hz [12,13]. As a result, the COSY spectrum does not show these correlations because of the very small value of the coupling constants. The SUPER COSY experiment suffers from sensitivity problems associated with J-tuning for such small J values [14,15]. Here we report results from direct observation of the base-H1′ J-coupling correlations in a 2D correlated spectrum of the dinucleotide CpG, making use of the recently discovered technique, J-scaled COSY [16]. We also show how this enabled unambiguous assignment of all the non-exchangeable protons in the molecule.

2. 2D NMR TECHNIQUES

The principles of 2D correlated spectroscopy have been described in the literature [11]. The cross-peaks in the COSY spectrum reflect J-coupling correlations and are made up of components which have anti-phase character (opposite signs) along both ω_1 and ω_2 axes. This anti-phase character becomes a particular disadvantage when the experiment is carried out under conditions of poor resolution, or when the coupling constants are small, since the cancellation of component intensities leads to poor cross-peak intensities. Recent developments, namely SUPER COSY [14] and J-scaled COSY [16], have overcome some of the limitations of the conventional COSY experiment arising from the anti-phase character of the cross-peak components. These employ the following pulse schemes:

SUPER COSY:

$$90°-t_1-\tau_1-180°-\tau_1-90°-\tau_2-180°-\tau_2-t_2$$

J-scaled COSY:

$$90°-t_1-\tau-180°-\tau-90°-\tau_2-180°-\tau_2-t_2$$

where t_1 and t_2 are the usual evolution and detection periods of 2D NMR spectroscopy and τ_1 and τ_2 are additional constant delay periods. τ is a variable delay which is incremented synchronously with t_1:

$$\tau = pt_1$$

The proportionality constant p determines the J-scaling factor η ($\eta = 1 + 2p$). The SUPER COSY experiment achieves refocussing and produces in-phase multiplet components when the delays τ_1 and τ_2 are optimally adjusted (for 2 spins τ_1 and τ_2 = 1/4 J). This produces intense cross-peaks, and at the same time, the diagonal peaks acquire anti-phase character and have lower intensities. However, in many cases and particularly for small J values (~1 Hz), the delay periods become too long (0.25 s) resulting in serious problems of sensitivity due to decay of the signal. On the other hand, in the case of the J-scaled COSY technique, the separation between the anti-phase components is increased along the ω_1 axis, and thus the

cancellation of component intensities in the cross-peaks is minimised. Since τ is now proportional to t_1, and hence is a much shorter delay compared to τ_1, there is a significant gain in sensitivity as compared to the SUPER COSY experiment, from the point of view of T_2^* loss. Along the ω_2 axis, however, there is no convenient way of J-scaling and a refocussing delay becomes necessary for the observation of small J correlations.

3. EXPERIMENTAL

Cytidylyl(2′–5′)guanosine was purchased from Sigma. 1D, 2D COSY, SUPER COSY and J-scaled COSY spectra of CpG in D_2O at 25°C were recorded on a Bruker AM-500 FTNMR spectrometer. For the COSY experiment 1024 data points were taken along t_2 and 256 along t_1 with quadrature detection and quadrature phase cycling. The data points along t_1 were zero filled to 512. The time domain data were multiplied by phase-shifted sine square bell and sine bell functions along t_2 and t_1, respectively, prior to respective Fourier transformations. The respective phase shifts were $\pi/64$ and $\pi/32$. For the SUPER COSY experiment 2048 data points along t_2 and 512 along t_1 were collected with quadrature phase cycling and quadrature detection. The data points along t_1 were zero filled to 1024. Fourier transformation was made following window multiplications using sine square bell and sine bell functions along t_2 and t_1 directions, respectively. The J-scaled COSY experiment was carried out with 1024 data points along t_2 and 256 along t_1 using single channel detection. The data points along t_1 were zero filled to 512. The time domain data were processed as in the SUPER COSY experiment.

In all the experiments the HDO signal was reduced by low-power continuous irradiation at all times except during the detection period. Chemical shifts were measured with respect to sodium 3-trimethylsilyl[2,2,3,3-^{2}H]propionate.

4. RESULTS AND DISCUSSION

Fig.2 shows the COSY and SUPER COSY spectra of CpG in D_2O recorded at 25°C. The cross-peaks show direct coupling correlations between

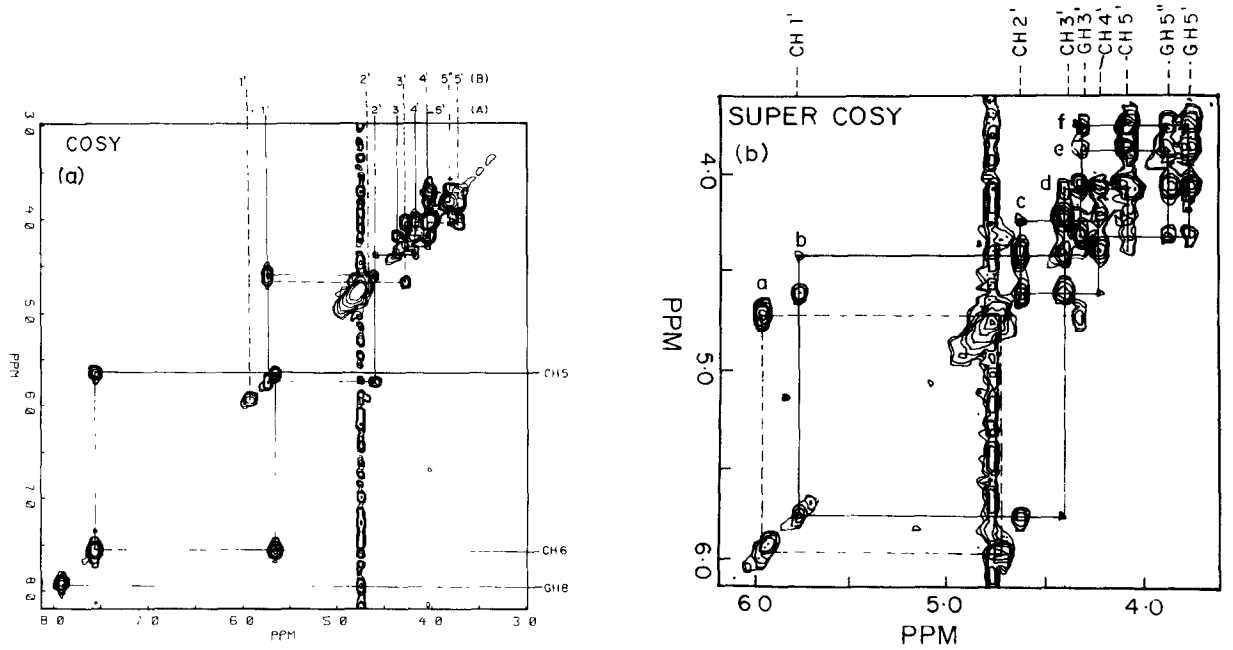

Fig.2. (a) COSY spectrum of CpG. (– – –) Coupling correlations in ring B, (——) coupling correlations in ring A. (b) SUPER COSY spectrum of CpG. (——) 4-bond coupling correlations (peaks b–f). H1′–H2′ coupling correlation of sugar ring B (peak a), not seen in 'a', is also observed (– – –). Identification of the protons is indicated. The delay periods τ_1 and τ_2 are both 0.05 s.

the protons of the 2 sugar rings A and B as indicated by the connectivities in the COSY spectrum of fig.2a. The cross-peak for H1′-H2′ of ring B is missing in the COSY spectrum, but is clearly seen in the SUPER COSY spectrum which is shown in fig.2b. This spectrum also shows a number of 4-bond correlations, namely CH1′-CH3′, CH2′-CH4′, CH3′-CH5′, GH3′-GH5′, and GH3′-GH5′′ within the sugar rings. Another 4-bond correlation, GH1′-GH3′, was also seen at lower levels, and these provided confirmation of the spin system connectivities shown in fig.2a. However, these spectra did not allow assignment of the spin systems to particular nucleotides in the molecule. The assignment indicated in fig.2b was obtained from the *J*-scaled COSY spectrum (fig.3a). In this spectrum the *J* value was scaled 6 times (p = 2.5) along the ω_1 axis and a ω_2-refocussing delay (τ_2) of 0.15 s was used. The spectrum shows GH8–GH1′ and CH6–CH1′ cross-peaks which are indicated by the connectivities drawn in. The CH6–CH1′ cross-peak lies very close to the CH6–CH5 cross-peak due to the close proximity of the CH5 and CH1′ chemical shifts. A vertical cross-section at the CH6 position (fig.3b) clearly shows 2 doublets corresponding to CH5 and CH1′ protons. Thus, sugar ring A was assigned to a cytosine nucleotide and ring B to a guanine nucleotide. In fig.3a, the diagonal peaks of GH8 and CH1′ are weak and that of CH5 is not visible. Their positions have been obtained by comparison with the COSY spectrum.

It is interesting to observe that the *J*-scaled COSY spectrum of fig.3 does not show the symmetry which one normally sees in correlated spectroscopy. The CH6–CH1′ cross-peak is seen only in the upper triangle of the spectrum while the GH8–GH1′ cross-peak is seen only in the lower triangle of the spectrum, the diagonal running from the lower left corner to the upper right corner separating the 2 triangles. This can be understood by following the path of coherence transfer and the linewidths in the 2 dimensions. In the upper triangle the cross-peak components will possess the linewidths of H1′ protons along the α_1 axis and those of base protons along the ω_2 axis. On the other hand, the converse holds true in the lower triangle. Thus, in the absence of sufficient refocussing along the ω_2 axis, the cross-peaks in the upper triangle would have better intensity if the

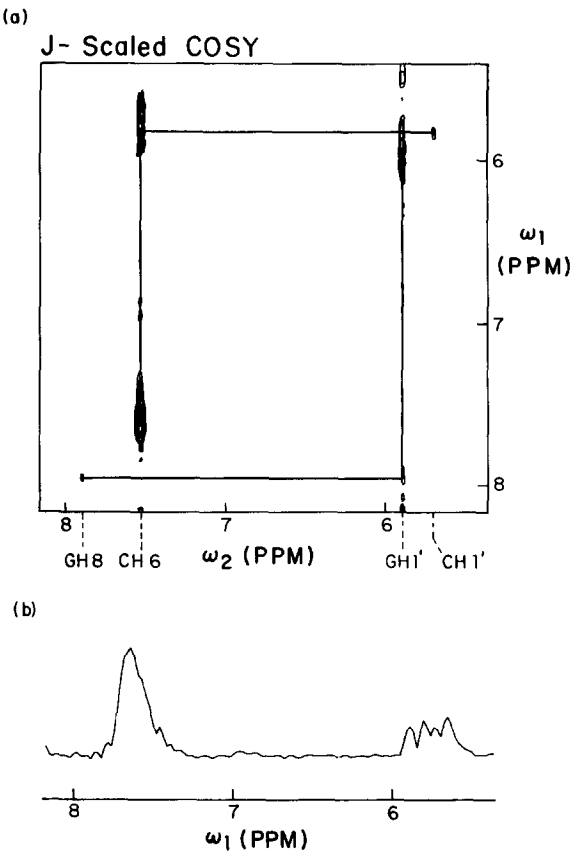

Fig.3. (a) *J*-scaled COSY with *J*-tuning along the ω_2 axis. 4-bond coupling correlations CH6-CH1′ and GH8-GH1′ are indicated by the connectivities drawn. Identification of the protons is given along the bottom. The diagonal peak for CH5 was not observed. *J* was scaled 6 times along the ω_1 axis and τ_2 was 0.15 s. (b) A vertical cross-section at the CH6 position. The 2 doublets constituting the cross-peak clearly indicate coherence transfer from CH6 to CH5 and CH1′.

base protons had sharper lines than the H1′ protons and similarly, the cross-peaks in the lower triangle would have better intensity if the H1′ protons had sharper lines than the corresponding base protons. The present data are consistent with the linewidths observed in the 1D NMR spectrum, namely, the linewidth of GH8 is larger than that of GH1′ and in the case of cytosine the H1′ resonance is broader than the H6 resonance. Similar arguments can be extended to the non-appearance of CH5 diagonal peaks, low intensity of GH8 and CH1′ diagonal peaks and the absence

of CH6-CH5 cross-peak in the lower triangle of the spectrum (this peak is actually weak and could be seen at lower levels). Here it must be considered that the τ_2 delay introduces anti-phase character in the diagonal peaks in the ω_2 direction. This results in the cancellation of intensities if the lines are broad.

For the purpose of observing such small J correlations as are shown here, the fixed delay period τ_2 of the pulse scheme can impose a certain limitation on the size of the molecule. In large systems, where T_2^* is very short, large delays cannot be afforded. Moreover, the refocussing delay parameter τ_2 may be properly adjusted for maximum elimination of anti-phase character, in the ω_2 direction, of the cross-peak components. In addition, increased linewidths due to short T_2^* values in large molecules lead to reduced cross-peak intensities because of the cancellation of anti-phase cross-peak components as illustrated above. However, this can be partially overcome by using larger data sizes in the t_2 direction, without significantly increasing the experimental time. The base type also seems to play a significant role and our preliminary experiments indicate that the H6–H1′ coupling constant in the pyrimidines is larger than the H8–H1′ coupling constant in the purines. We generally feel that this strategy of assignment would be feasible in moderately large systems, depending upon the characteristic relaxation times. Single-stranded nucleic acids may be much more amenable than double-stranded nucleic acids, because of higher mobilities and consequently sharper resonances. In adverse conditions of linewidths, larger quantities of material may be required to improve the sensitivity. When material is a limitation, extensive signal averaging may have to be performed. Considering all these practical aspects, we may speculate that with optimum experimental parametrization, oligonucleotides about 10–15 units long may be studied by the procedure described in this paper.

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