Journal of Biomolecular NMR, 1 (1991) 205–208
ESCOM

J-Bio NMR 014

A frequency-time domain 3D COSY-*J* technique for measurement of ^{1}H-^{31}P coupling constants in oligonucleotides

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Received 2 January 1991
Accepted 5 March 1991

Keywords: 3D COSY-*J*; H-P coupling constants; Oligonucleotides

SUMMARY

A frequency-time domain 3D NMR technique has been developed for measurement of heteronuclear coupling constants in oligonucleotides employing a combination of COSY and *J*-resolved techniques. The method employs frequency-selective excitation to generate the ω_1 axis and 2D FT to generate the ω_2 and ω_3 axes. The procedure yields high resolution, especially along the ω_1 axis. The technique is demonstrated on a dinucleotide.

Heteronuclear ^{1}H-^{31}P coupling constants constitute an important input for characterisation of backbone conformations of oligonucleotides (for reviews see Govil and Hosur, 1982; Gorenstein et al., 1983). H3′-P coupling constants provide information about the ε or C4′-C3′-O3′-P torsion angle, while (H5′,H5″)-P *J* values provide information about the β or C4′-C5′-O5′-P torsion angle along the nucleic acid backbone. Consequently, several experimental strategies based on 2D-*J* and 2D-correlated techniques have been devised for measuring these coupling constants (Sklenar and Bax, 1987; Hosur et al., 1988, 1990). These coupling constants have also been obtained indirectly by simulation of cross-peak patterns in different types of homonuclear *J*-correlated spectra (Chazin et al., 1986; Gochin et al., 1990). Our suggestion was to use homonuclear ^{1}H-^{1}H 2D *J*-resolved spectroscopy, in which the heteronuclear coupling is retained along the ω_2 axis, and thus appears in the ω_2 projection of the tilted *J*-resolved spectrum. As with other methods, the success of this experiment depends on the chemical shift dispersion of the protons coupled to the phosphorous nucleus, i.e., the H3′ protons for ε and H5′, H5″ for β.

Experimentation with several DNA segments in our laboratory and elsewhere have indicated

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that about 50–60% of the time, the dispersion of H3′ protons is not adequate for unambiguous measurement of ^{1}H-^{31}P coupling constants. The situation is worse for the H5′ and H5″ protons. In this communication, we have tried to overcome this difficulty by exploiting the chemical-shift dispersion of other protons scalarly coupled to the protons of interest. The basic idea is demonstrated with the simple example of a dinucleotide.

Figure 1 shows the pulse sequence for the suggested frequency-time domain 3D experiment. 90^{s} is a selective pulse generated by a DANTE pulse train (Morris and Freeman, 1978). By systematically incrementing the delay between the individual pulses in the pulse train, the corresponding excitation side-band frequency can be systematically decremented. In this way, frequency-selective excitation can be performed *in a controlled manner*, and this is used to generate the ω_1 axis of the 3D spectrum. The selectively excited nucleus is then allowed to evolve freely for a period τ and then the coherence is transferred to another nucleus scalarly coupled to the excited nucleus. This nucleus then evolves during t_2 and t_3, and data is acquired during t_3. The 180° pulse in the centre refocusses chemical shifts. 2D Fourier transformation of the data collected as a function of t_2 and t_3 generates the ω_2 and ω_3 axes of the 3D spectrum. The last part of the pulse scheme is essentially that of 2D *J*-resolved spectroscopy. The phase cycling of the pulses is adjusted in such a way that only multiplets originating from the selectively excited nucleus are retained during the t_2 and t_3 periods. Thus, the 3D planes orthogonal to the ω_1 axis will consist of selectively filtered regions of the 2D *J*-resolved spectrum.

In the above experiment, the frequency step (or resolution) and frequency selectivity are determined by the delay increment in the DANTE pulse train and the total length of the pulse train, respectively. Because of the inverse relationship between the delays and the excitation frequencies, both the frequency step and the selectivity of excitation keep changing through the course of the experiment. Consequently, calibration and optimisation of parameters along ω_1 are different compared to ω_2 and ω_3.

The proposed technique is actually a combination of all-time-domain 3D experiments (Vuister and Boelens, 1987; Griesinger et al., 1987, 1989) and time-frequency-domain pseudo-3D experiments which do not contain any evolution periods (Davies et al., 1988). The all-time-domain experiments are highly time-consuming, particularly when one is interested in a small region of

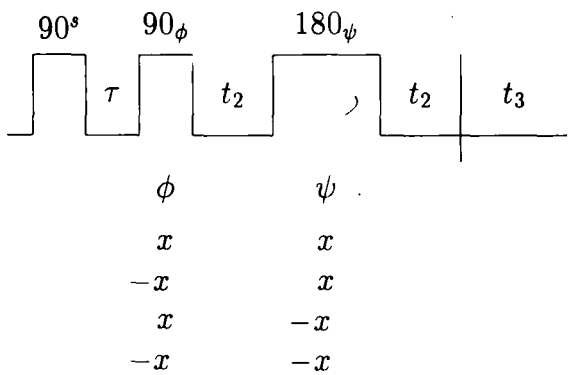

Fig. 1. Pulse sequence and phase cycles for the proposed 3D COSY-*J*-resolved experiment. 90^{s} is a selective pulse generated using the DANTE pulse train for generating the ω_1 axis. τ is a fixed delay. The data is co-added in the memory.

the complete spectrum. For purposes such as coupling constant measurement, the frequency-time-domain experiments are very suitable, since the available experimental time can be optimised for recording selected regions of the spectrum with high resolution.

Figure 2a shows an application of the suggested experiment to a ribo-dinucleotide 5'-GpC-2', in which the phosphodiester linkage is between the 2' position of G and 5' position of C, unlike the usual 5'-3' connectivities. The sample is well-suited for the present purpose because of degenerate chemical shifts of the GH2' and CH2' protons at 35°C and pH 7.0. In the normal 2D *J*-resolved spectrum shown in Fig. 2b, the H1' and CH5 doublets are clearly visible, but the multiplets of GH2' and CH2' are not discernible at all. In fact, GH2' should appear as a doublet along the δ axis because of its coupling with ^{31}P, whereas CH2' should be a singlet. These features are clearly seen in the two planes in the 3D spectrum in Fig. 2a, which was recorded with selective excitation of the H1' protons along the ω_1 axis. It may be noted that the digital resolution along ω_3 in Fig. 2a is *half* the digital resolution along the corresponding δ axis in Fig. 2b. From the GH1' plane, the heteronuclear coupling constant can be estimated to be $\sim$ 10 Hz.

In the above example, spin-selective filtering was achieved by selective excitation of the H1' protons which are well resolved. When there is a greater degree of overlap, systematic incrementation of the ω_1 excitation frequency will have to extend over a selected region. The experiment then resembles a regular all-time domain 3D experiment, but with 2D Fourier transformation. Besides, in natural DNA or RNA systems in which the phosphorus is attached at the 3' and 5' positions, frequency-selective excitation will have to be applied to the 2' protons for 3' selection and to the 4' protons for 5' selection. The H2' protons show good chemical-shift dispersion in DNA and thus

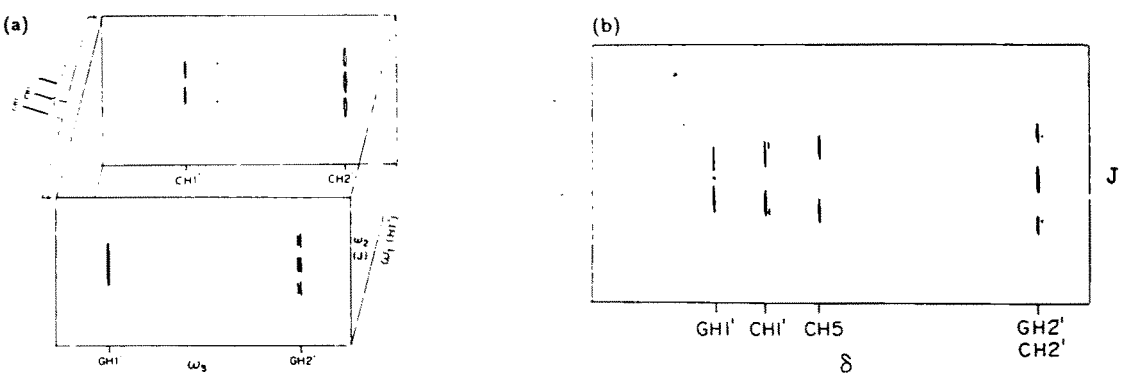

Fig. 2. (a) Slices of the 3D *J*-resolved spectrum of 5'-GpC-2', containing peaks originating from the excitation of H1' (ω_1) protons using the pulse sequence shown in Fig. 1. The spectrum consists of two 2D planes originating from selective excitation of GH1' and CH1' protons as indicated by arrows on the left top. Thirty-two t_2 experiments were performed with 4096 t_3 points each. The DANTE pulse train consisted of 45 pulses of 0.3 μs duration each. The interval between the pulses was different for the two planes and the total duration of the DANTE pulse was 36.7 ms for the GH1' plane and 40.3 ms for the CH1' plane. τ was set to 30 ms. The carrier was placed about 100 Hz upfield to the low-frequency end of the spectrum, and the spectral width along ω_3 was twice the normal spectral width. Digital resolutions are: $\omega_3 = \omega_2 = 2.03$ Hz/pt. (b) Region of the 2D *J*-resolved spectrum of 5'-GpC-2' showing overlap of CH2' and GH2' peaks. Thirty-two t_1 experiments were performed with 4096 t_2 points each. The sample concentration is approximately 5 mM in ^2H_2O, pH 7.0. Because of low S/N on our spectrometer ($\sim$ 40 on standard ethyl benzene test samples) which is more than seven years old now, we had to collect 48 scans for each free-induction decay in both (a) and (b). With modern spectrometers, four scans should be sufficient for each FID with the above concentrations of the sample.

permit filtering of 3′ doublets (due to H3′-P coupling) in the different planes of the spectrum. Each plane may of course contain more than one 3′ doublet, depending upon the degeneracy in the H2′ chemical shifts and selectivity of excitation. We believe that the technique will be useful in measuring the much needed H3′-P coupling constants in nucleic acids in a larger number of systems than has been possible before. The H5′-P couplings, however, remain difficult to obtain because of the severe overlap of H4′, H5′ and H5″ chemical shifts.

The technique described in this communication is fairly simple, and can be performed on all spectrometers equipped with 2D capabilities. Specific regions of the spectrum can be recorded with high resolution and in a short period of time (a few hours). A disadvantage, however, is that the spectra will have to be recorded in absolute-value mode because of mixed-phase lineshapes. Although the pulse sequence described is for homonuclear systems, it is easy to extend the ideas to heteronuclear experiments.

ACKNOWLEDGEMENTS

Facilities provided by the 500 MHz FTNMR National Facility supported by the Department of Science and Technology, Government of India, are gratefully acknowledged.

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