# TWO-DIMENSIONAL J-RESOLVED $^{1}$H n.m.r. SPECTROSCOPY FOR STUDIES OF BIOLOGICAL MACROMOLECULES

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SUMMARY: The technique of two-dimensional J-resolved $^{1}$H n.m.r. spectroscopy has been extended to handle the very wide spectra of proteins and other macromolecules at 360 MHz. The potential of the method to resolve and assign individual spin multiplets in the complex spectra encountered in structural studies of biopolymers is illustrated with some experiments with amino acids and with a protein, the basic pancreatic trypsin inhibitor.

The potential of high resolution nuclear magnetic resonance (n.m.r.) for structural studies in biological systems depends critically on the ability to resolve and assign individual spin multiplets in the inherently complex spectra of biological macromolecules (1). In addition to using high polarizing magnetic fields, suitable digital filtering techniques (2-4) and the use of spin echo experiments (5) have recently resulted in markedly improved spectral resolution for conventional $^{1}$H n.m.r. spectra of proteins. Here, a new concept is applied which promises to greatly enhance the resolution in the $^{1}$H n.m.r. spectra of biopolymers and to facilitate assignment of individual spectral components, i.e. two-dimensional J-resolved $^{1}$H n.m.r. spectroscopy (6-9) at high field.

The present paper describes for the first time two-dimensional high field $^{1}$H n.m.r. experiments at 360 MHz. With the versatile software written to handle the large data matrices required for studies of macromolecules, two-dimensional $^{1}$H n.m.r. can now be applied to greatly simplify the interpretation of the spectra of biopolymers. The initial experiments with aqueous solutions of amino acids and proteins clearly illustrate the potential of the method for resolving and assigning individual spin multiplets in complex spectra of biological materials, where extensive overlap of resonance lines is typically observed in conventional one-dimensional spectra.

MATERIALS AND METHODS: The basic method of 2-dimensional (2 D) J-resolved n.m.r. has been described in Ref. (6). Its principles shall briefly be summarized in this section. The technique is an example of the general class of 2 D resolved spectroscopy techniques (6-9); in this case, the multiplet splitting is utilized to obtain a 2 D spread of the proton magnetic resonance spectrum.





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The technique requires the performance of N two-pulse spin echo experiments of the type $90^{\circ}$ - $k\tau$ - $180^{\circ}$ - $k\tau$, with k = 0, 1, ...(N-1). Due to the refocusing effect of the $180^{\circ}$ pulse, an echo appears with its maximum at time $k\tau$ after the $180^{\circ}$ pulse. Of the second half of each echo, M equidistant sample values are recorded, defining the M by N data matrix $(S_{k\ell})$ k = 0,...(N-1); $\ell$ = 0,...(M-1). Each of the N echo traces (corresponding to a row of the data matrix) is then Fourier-transformed to separate the contributions originating from the various resonance lines.

It is well-known that for weak spin-spin coupling the echo amplitude is independent of the chemical shift. The echo amplitude and correspondingly the separated signal amplitudes are thus modulated exclusively by the multiplet splittings. This modulation is utilized to differentiate between the resonance lines with different multiplet splittings by means of a second Fourier transformation, this time transforming the columns of the data matrix. This produces, finally, a 2 D spectrum, $S(\omega_1,\omega_2)$. The coordinates of each signal peak in the 2 D spectrum are given in the $\omega_2$-direction by the resonance frequency of the conventional one-dimensional spectrum and in the $\omega_1$-direction by the corresponding multiplet splitting. The resonance intensities are equal to those in the one-dimensional spectrum.

Applications of 2 D spectroscopy to protein n.m.r. require the handling of particularly large data matrices. In the present configuration, our equipment readily handles up to $10^6$ data points in the time domain. The spectra in Figs. 2 and 3 were computed from a data matrix with N = 128 and M = 8192, that in Fig. 4 with N = 64 and M = 8192. Additional experimental details are given in the figure captions.

RESULTS: To explain some principal features of the high field 2 D $^1$H n.m.r. spectra, we first consider the resonances observed in a $D_2O$ solution of an equimolar mixture of the five amino acids alanine, isoleucine, methionine, tyrosine and histidine. For this sample the resonances of the aromatic protons of His and Tyr are already well resolved in the region from 6 to 8 ppm of the conventional 360 MHz spectrum (Fig. 1 A). In the 2 D spectrum, since the two singlet resonances of His are not modulated by a J coupling, these lines are not affected by the 2 D spread in the $\omega_1$-direction and hence appear with their respective chemical shifts on the $\omega_1$ = 0 line (Fig. 2). For the doublets of Tyr, the modulation results, after Fourier transformation, in displacements in the $\omega_1$-direction by $\pm \pi J_{AB}$ rad/sec relative to the $\omega_1$ = 0 line.

In the high field spectral region, Met gives rise to a singlet methyl resonance at 2.1 ppm (Fig. 1 A) which is located on the $\omega_1$ = 0 line in the 2 D spectrum (Fig. 2). The two component lines of the methyl doublet resonance of Ala at 1.2 ppm are displaced in the $\omega_1$-direction, analogous to the doublets of Tyr. Ile gives rise to a doublet and a triplet methyl resonance which partially overlap at 0.9 ppm in the conventional spectrum (Fig. 1 A). In the 2 D spectrum these two multiplets are fully resolved and complete assignments of the two multiplets are readily obtained. The appearance of the doublet corresponds to

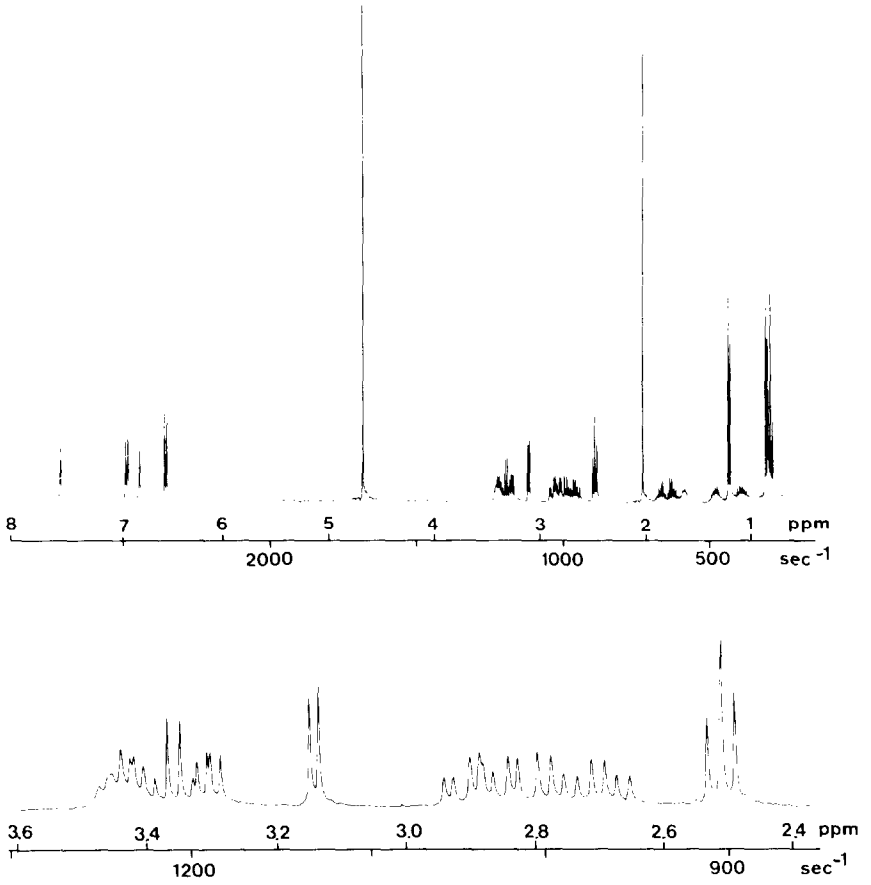


Fig. 1 A. Fourier transform $^{1}H$ n.m.r. spectrum at 360 MHz of a $D_2O$ solution containing 0.1-M of each of the five amino acids Ala, Ile, Met, Tyr and His, pD = 10.5, T = 25°. Chemical shifts relative to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) are indicated both in Hertz ($sec^{-1}$) and in parts per million (ppm). The spectrum was computed from 8192 data points. B. Expanded representation of the spectral region from 2.4 to 3.6 ppm.

that described above for Tyr and Ala; for the triplet, the center line is at $\omega_1 = 0$ and the peripheral lines are displaced by $\pm 2\pi$ J rad/sec in the $\omega_1$ direction. It is essential to recognize that, as a result of the spread in a second dimension, all lines belonging to a particular multiplet will be alined on a straight line which forms an angle of 45° with the $\omega_2$-axis and intersects the $\omega_1 = 0$ line at the chemical shift of the proton considered. This is not well visible from Fig. 2 as the scales of the two axes differ by two orders of

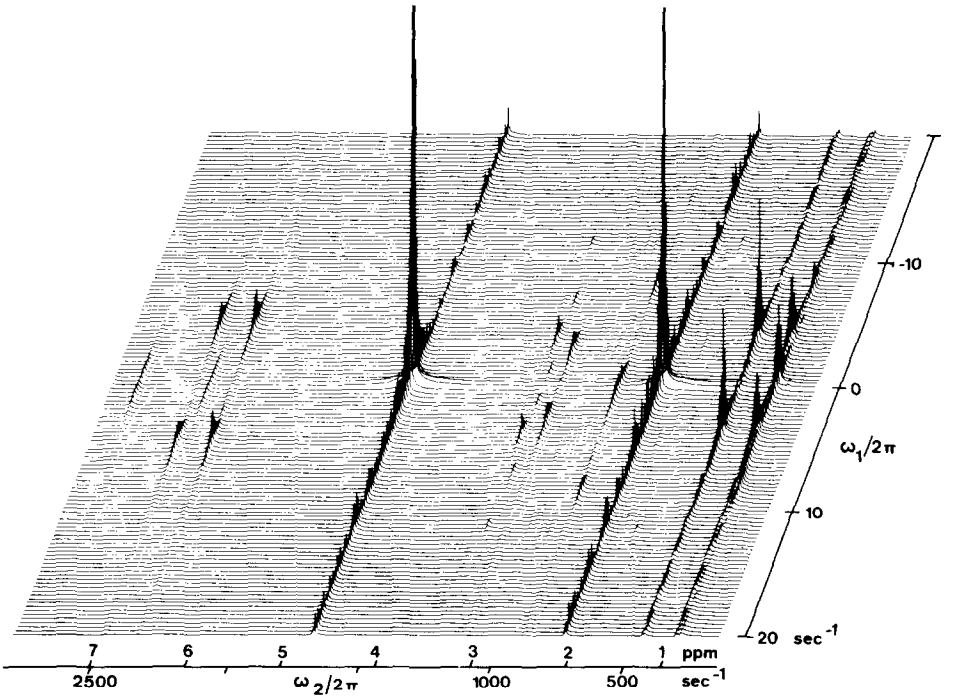


Fig. 2 Two-dimensional J-resolved 360 MHz $^1$H n.m.r. spectrum of the amino acid mixture of Fig. 1. An absolute value spectrum is shown. The spectrum was computed from 128 x 8192 data points corresponding to 128 single echoes represented by 8192 sample values. The two frequency axes are calibrated in Hz, for the $\omega_2$-axis the corresponding ppm scale relative to DSS is also indicated.

magnitude. The expanded spectra of Figs. 3 and 4 provide a somewhat better demonstration of this basic feature of 2 D J-resolved spectra.

A nice illustration of the power of 2 D NMR to resolve and assign individual spin multiplets is provided by the spectral region from 2.4 to 3.6 ppm in Fig. 1 A, which is represented on an expanded scale in Fig. 1 B. This region contains 10 spin multiplets, some of which mutually overlap in the one-dimensional spectrum (Fig. 1 B). In the expanded representation of the corresponding region of the 2 D spectrum in Fig. 3, all the resonance lines are well separated and the spin multiplets can readily be assigned. In the order of increasing chemical shift in this spectral region, we first have a two-proton triplet of the γ-methylene protons of Met (1). Next, there are four one-proton multiplets of the β-methylene protons of Tyr and His, each of which corresponds to a doub-

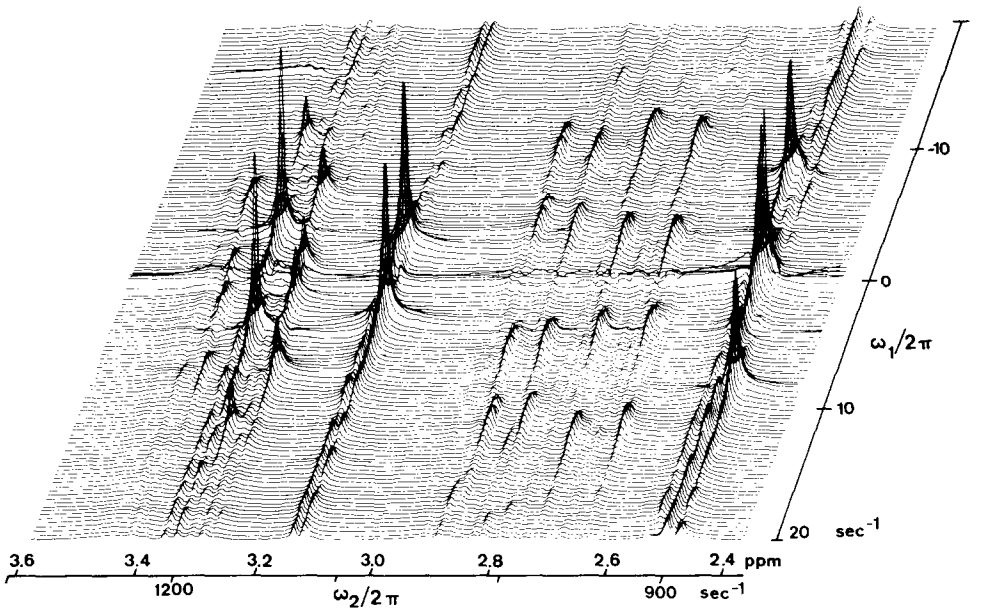


Fig. 3 Expanded representation of the spectral region from 2.4 to 3.6 ppm in the two-dimensional spectrum of Fig. 2, corresponding to the 1-dimensional expanded spectrum of Fig. 1 B.

let of doublets. The α-proton doublet resonance of Ile was already well resolved in the conventional spectrum. The remaining four α-proton resonances are a doublet of doublets for Met, a quartet for Ala, a doublet of doublets for Tyr and a doublet of doublets consisting of rather broad lines for His.

Fig. 4 shows the high field region from 0.5 to 1.7 ppm of the 1 D and 2 D spectra of the basic pancreatic trypsin inhibitor (BPTI). This protein consists of one polypeptide chain with 58 amino acid residues, including 6 Ala, 1 Val, 2 Leu, 2 Ile and 3 Thr, which give rise to 19 methyl doublet and triplet resonances at high field. These methyl resonances had previously been assigned to the different types of aliphatic amino acid side chains (10). In the 2 D spectrum, the components of the individual multiplets, which are partially overlapped in the 1 D spectrum, can readily be assigned. Between 1.61 and 1.04 ppm there are eight doublet resonances 2 - 9 corresponding to Ala and Thr. It is worth noting that the narrow lines of resonance 5, which corresponds to the C-terminal Ala 58 (10), are very prominent after digital resolution enhancement in both the 1 D and 2 D spectrum. Between 1.0 and 0.71 ppm, there are the eight doublets 10 - 17 of Val, Leu and Ile. In the presentation of Fig. 4, the pairs

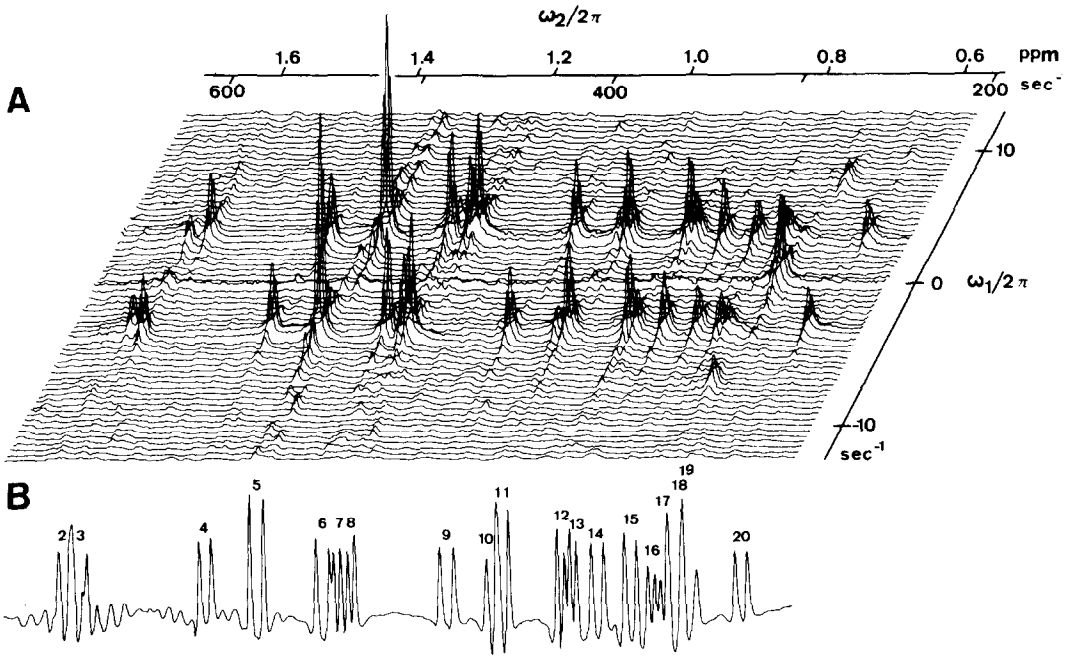


Fig. 4 A. Two-dimensional J-resolved 360 MHz $^1$H n.m.r. spectrum of a 0.01-M solution of the basic pancreatic trypsin inhibitor in $D_2O$, pD = 4.5, T = 60°. The figure shows the expanded region from 0.5 to 1.7 ppm of a spectrum computed from 64 x 8192 data points. Prior to the Fourier transformations the FID's were multiplied with an increasing exponential to reduce the line widths (1,2). B. One-dimensional $^1$H n.m.r. spectrum obtained after digital filtering with the sine bell routine (4). The individual methyl resonances are indicated by the numbers 2 ~ 20 (10).

of neighboring doublets 10 and 11, 12 and 13, and 16 and 17 appear partially overlapped, but can be well separated in a more expanded plot. The two triplet resonances of Ile, 18 and 19, have nearly identical chemical shifts (10) and appear strongly overlapped in Fig. 4, while the doublet 20 at the high field end, which comes from Ala or Thr (10) is well separated at 0.59 ppm. It is apparent also in this figure that the individual component lines of the multiplets can readily be assigned and the coupling constants J obtained by inspection of the 2 D spectrum.

DISCUSSION: Two-dimensional J-resolved $^1$H n.m.r. spectroscopy is a very general technique to unravel complicated n.m.r. spectra. Conceptually, it appeared particularly suited for the simplification of the analysis of high field $^1$H n.m.r.

spectra of proteins. This is to a large extent born out by the results presented in this paper. These initial experiments indicate that the utility of n.m.r. for studies of biopolymers will be greatly enhanced by the use of 2 D techniques.

The following features of 2 D J-resolved n.m.r. spectra appear to be of particular relevance for biological applications: (i) As mentioned in Section 3, multiplet lines in a 2 D spectrum are alined along straight lines with $45^{\circ}$ slopes relative to the $\omega_2$ axis. It is, therefore, possible to project the 2 D spectrum along this distinguished direction to eliminate the multiplet splitting and, effectively, to obtain completely homonuclear decoupled $^{1}H$ n.m.r. spectra (6). Similarly, spectra with reduced multiplet separations, corresponding in appearance to the residual couplings obtained in off-resonance $\{^{1}H\}^{13}C$ n.m.r. (1), may be obtained. (ii) 2 D experiments, in general, are time-consuming. It should be emphasized, however, that the additional time required reflects itself in an enhanced sensitivity. Indeed, within a given performance time it is possible to obtain a 2 D spectrum with almost the same sensitivity as that achievable for a one-dimensional spectrum (8). (iii) Since the appearance of simple features in the 2 D J-resolved n.m.r. spectra depends on the weak coupling assumption, it is essential to combine the use of this technique with the application of the highest available magnetic field strength. For strong coupling, new characteristic lines will appear in addition to the higher order features already observed in the conventional spectra (7), which can complicate the analysis of the 2 D spectra.

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