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SELECTIVE SPIN DECOUPLING IN THE J-RESOLVED TWO-DIMENSIONAL ^{1}H n.m.r. SPECTRA OF PROTEINS

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SUMMARY: Methods were developed where selective homonuclear spin decoupling is used for the identification of the spin systems of individual amino acid residues in J-resolved two-dimensional high field ^{1}H n.m.r. spectra of proteins. Experiments with the basic pancreatic trypsin inhibitor are shown to illustrate the practical application of these new techniques.

High resolution nuclear magnetic resonance (n.m.r.) is the only hitherto known method which can provide a many-parameter characterization of biopolymer conformation in solution (1). It has thus become an important complimentary technique to X-ray crystallography for studies of protein conformation (2). However, even after the introduction of very high field magnets, the full use of the potential of n.m.r. in this field still depends critically on the ability to resolve and assign numerous resonance multiplets in the crowded ^{1}H n.m.r. spectra of proteins. Recently we have demonstrated that greatly improved resolution can be obtained in J-resolved two-dimensional (2D) ^{1}H n.m.r. spectra of proteins at high field (3-5). The present note describes our first selective homonuclear spin decoupling experiments in 2D ^{1}H n.m.r., which further simplify the analysis of protein spectra.

In the analysis of the ^{1}H n.m.r. spectrum of a protein, the identification of the complete spin systems of individual amino acid residues is a fundamental first step. These resonance identifications result primarily from spin decoupling experiments (1). However, since at least the α-proton of each spin system is usually located in a very crowded spectral region, the interpretation of double resonance phenomena in the conventional one-dimensional spectra is often not unambiguous, even with the use of resolution enhancement and difference spectroscopy techniques (6). With J-resolved 2D n.m.r., individual α-proton resonance multiplets can be resolved in small and medium sized proteins, and hence the combination with spin decoupling has a greatly improved potential for identification of the spin systems of individual amino acid residues.

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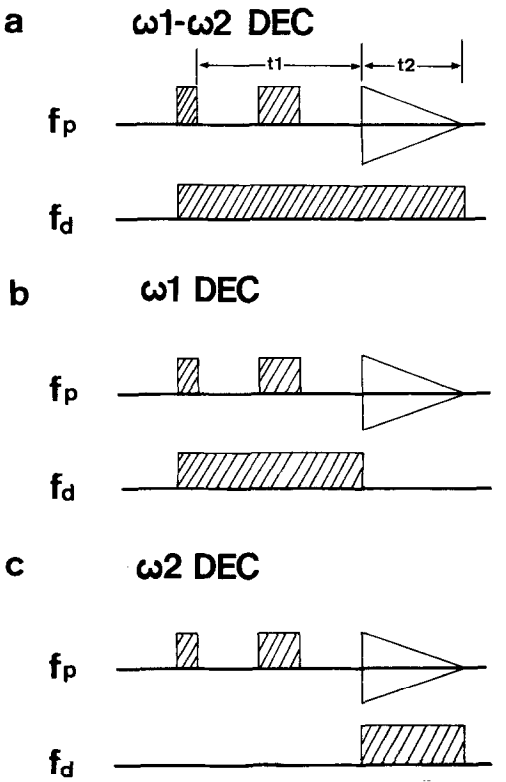

Fig. 1 Schematic presentation of three types of selective homonuclear spin decoupling experiments in two-dimensional ^1H n.m.r. spectroscopy. The top trace of experiment a, (f_p), outlines the typical 90° - τ - 180° - - τ pulse sequence used to obtain a J-resolved 2D ^1H n.m.r. spectrum (3-5,7,8), where t_1 is the evolution period and t_2 the detection period. In experiment a, which was used to obtain the data in Figs. 3 and 4, the decoupling field f_d is applied during the entire experiment, in experiments b and c only during t_1, or t_2, respectively. Note that in reality the amplitude of the field f_d is much lower than that of f_p.

MATERIALS AND METHODS: J-resolved 2D n.m.r. is an example of the general class of 2D resolved spectroscopy techniques (7,8). Its principles were described previously (3-5,7,8). Suffice it here to recall that the fundamental experiment consists of an evolution period of length t_1, in the middle of which a refocusing 180° pulse is applied, and a detection period with the time variable t_2 (Fig. 1). The additional time variable needed to obtain a two-dimensional spread of the spectrum is obtained by repeating this experiment N times with different lengths t_1 of the evolution period. The spectrum in the corresponding two-dimensional frequency space, (ω_1,ω_2), is then obtained by a two-dimensional Fourier transformation.

As is shown schematically in Fig. 1, three different types of spin decoupling may be distinguished. When the double resonance irradiation f_d is applied only during the evolution period (Fig. 1b), the multiplets are collapsed in the ω_1-direction. When f_d is applied only during the detection period (Fig. 1c),

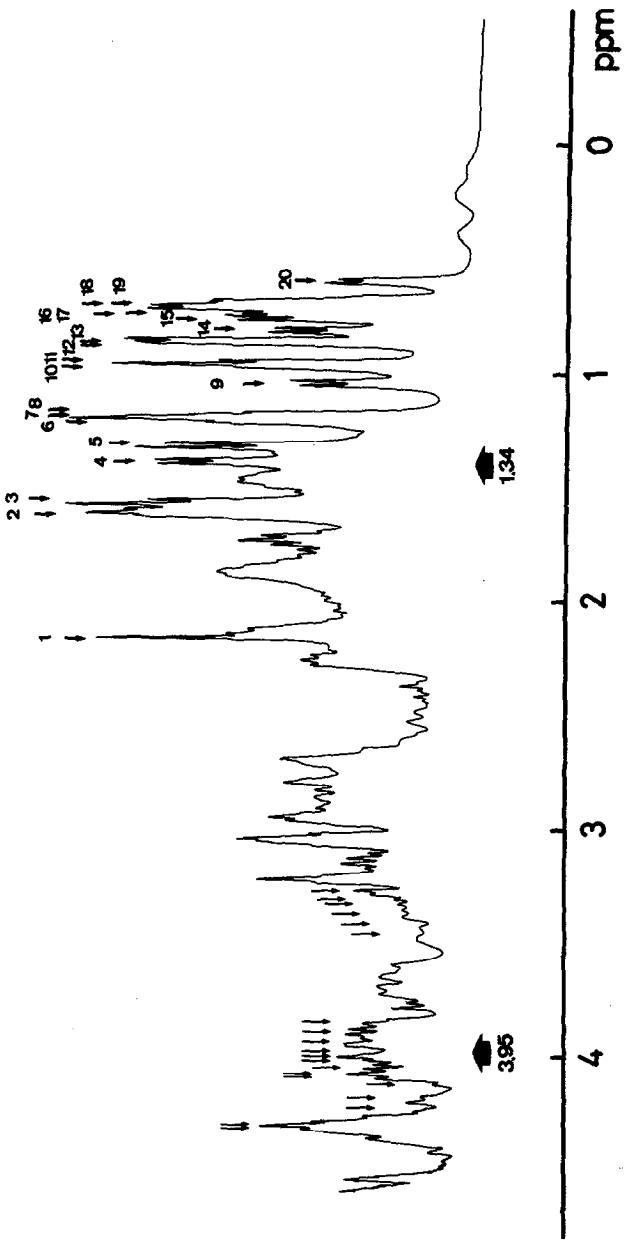

Fig. 2 Spectral region from 0 to 4.5 ppm of the one-dimensional Fourier transform ^{1}H n.m.r. spectrum of an 0.01 M solution of BPTI in $^{2}H_2O$, $p^{2}H = 4.5$, $T = 53^{\circ}$. The two bold faced arrows indicate the positions where the decoupling field f_d (Fig. 1a) was applied in the experiments of Figs. 3 and 4. The sharp arrows indicate the chemical shifts where the cross sections in the Figs. 3 and 4 were obtained. The methyl resonances are numbered in the order of the chemical shifts (6).

the multiplet splittings are eliminated in the ω_2-direction. When f_d is applied during both periods (Fig. 1a), the couplings are eliminated along ω_1 and ω_2. The theory for these three experiments has been worked out and it was shown that each of the three techniques can in principle provide the desired information (9). However, when working with complex molecules, informative presentation of the 2D spectra is a very important factor (5). With regard to this practical aspect, the experiment of Fig. 1a appears to be superior to experiments b and c, as it provides maximum simplification of the spectrum.

The basic pancreatic trypsin inhibitor (BPTI, Trasylol ^{R}) was obtained from the Farbenfabriken Bayer A.G. ^{1}H n.m.r. spectra at 360 MHz were recorded on a Bruker HXS 360 spectrometer, using a new improved version of our previously described software (3,5). The J-resolved 2D ^{1}H n.m.r. spectra were obtained from 64x8192 data points in the time domain. The digital resolution on the J-axis is 0.3 Hz (Figs. 3 and 4). The accumulation time per spectrum was approximately 6 hr.

RESULTS: In Figs. 2-4, experiments with the basic pancreatic trypsin inhibitor (BPTI) are used to illustrate spin decoupling in J-resolved 2D ^{1}H n.m.r. spectra. BPTI is a small globular protein with molecular weight 6'500. For the present study, it is important to point out that BPTI contains 20 methyl groups and that it is outstandingly stable with respect to thermal denaturation, so that the spectra recorded at 53° correspond to the native form of the protein. The 20 methyl resonances in the ^{1}H n.m.r. spectrum were previously identified and individually assigned (6,10).

The following remarks on the presentation of 2D spectra may be useful for a better understanding of the spectra in Figs. 3 and 4. In a J-resolved 2D n.m.r. spectrum represented in a (ω_1,ω_2) frequency plane, all components of a particular multiplet are aligned on a straight line which forms an angle of 45° with the ω_2-axis and intersects the ω_1=0 line at the chemical shift of the proton considered (3,5,7). The spin-spin coupling is thus manifested exactly equally in the directions of the ω_1 and the ω_2 axes. Provided that the same decoupling is applied for ω_1 and ω_2, which is the case in the experiment of Fig. 1a, identical residual spin-spin couplings will prevail along the two axes and the multiplet will still be located exactly on the 45° line. As a consequence, after a tilt by 45° to obtain a (δ,J)-presentation of the spectrum (5), the residual couplings are aligned on straight lines perpendicular to the chemical shift axis. Therefore the results of a spin decoupling experiment can be presented in the form of cross sections perpendicular to the chemical shift axis in the (δ,J) spectrum, which were suggested previously as a particularly suitable means for the presentation of complex 2D spectra (5).

Fig. 2 shows the spectral regions of the one-dimensional Fourier transform spectrum of BPTI which were used for the 2D spin decoupling experiments. Fig. 3

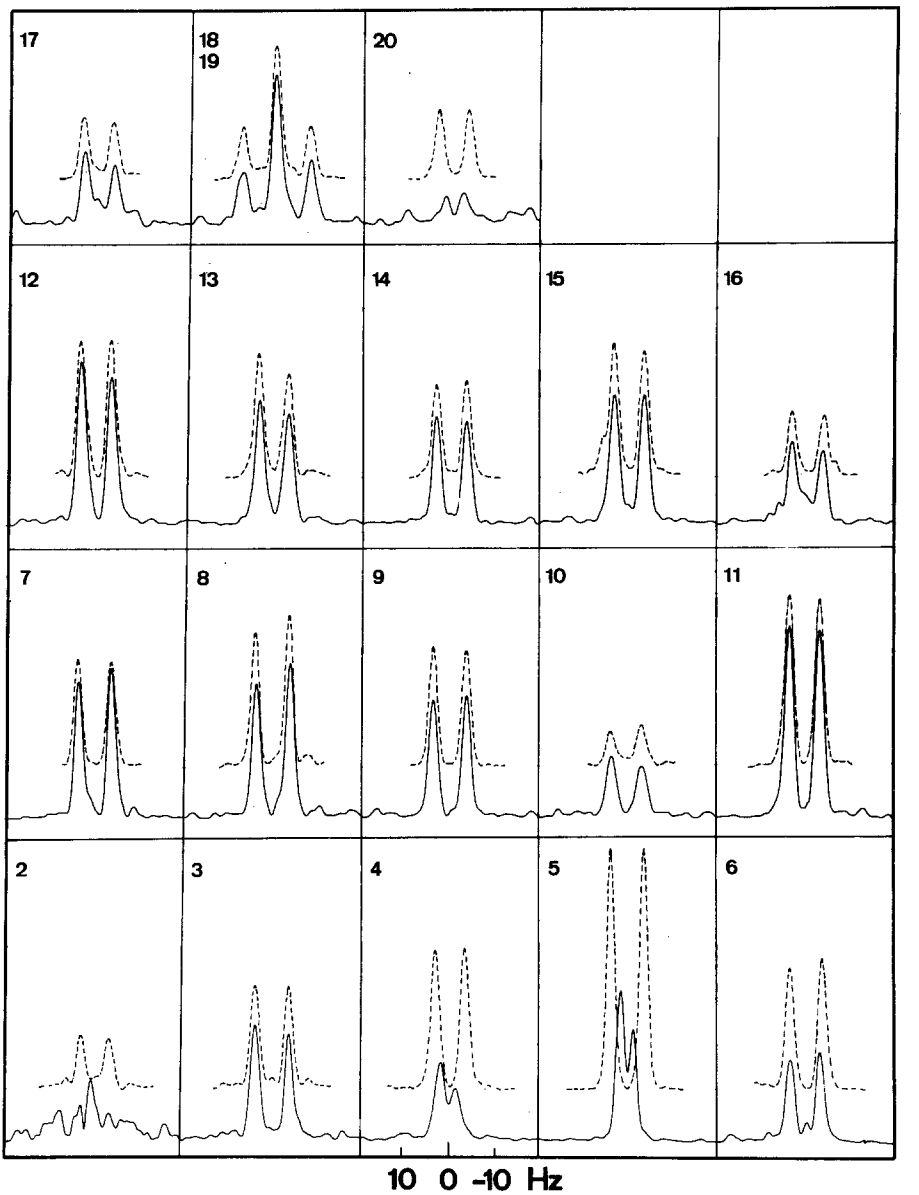

Fig. 3 Cross section representation of the high field region of the J-resolved 2D ^{1}H n.m.r. spectrum of the BPTI solution of Fig. 2, showing the multiplets of 19 of the 20 methyl groups in this protein. The numbers refer to the chemical shifts where the cross sections have been obtained (Fig. 2). The broken lines show the recently published multiplets obtained without double irradiation (5), the solid lines those obtained with spin decoupling at 3.95 ppm (Fig. 2). The n.m.r. parameters and individual assignments for the methyl groups in BPTI were previously reported (6,10).

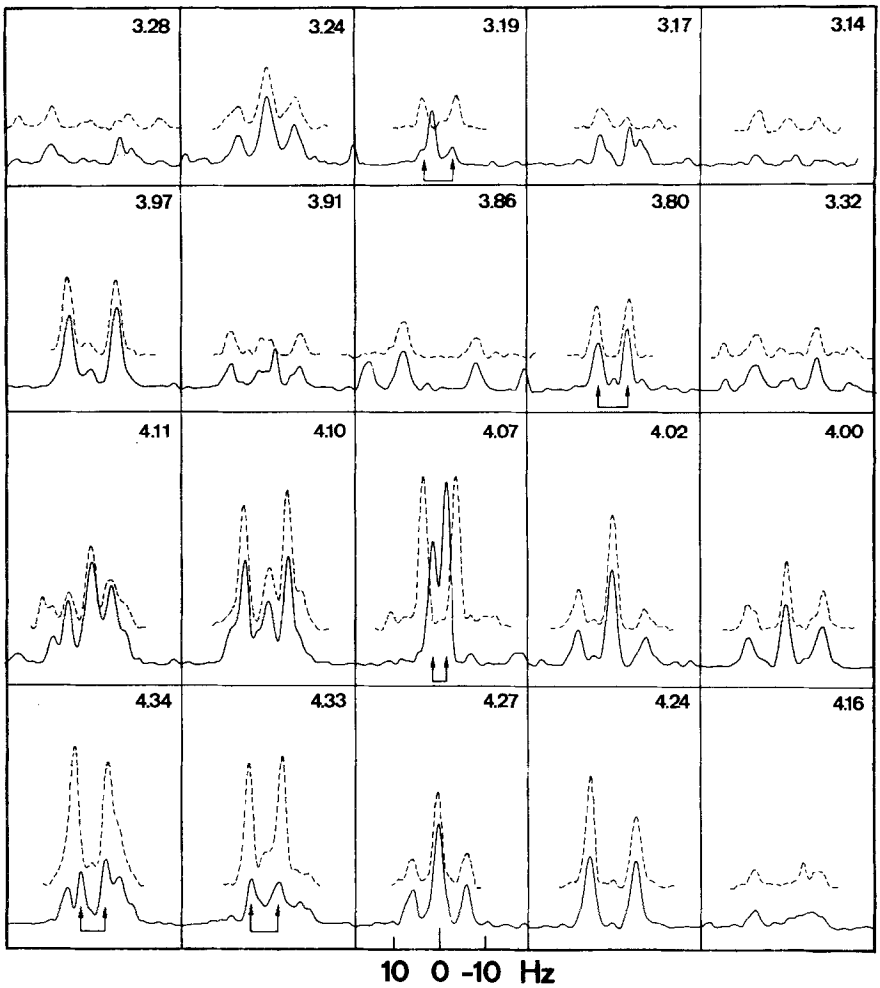

Fig. 4 Cross section representation of 20 multiplets in the region from 3.0 to 4.5 ppm of the J-resolved 2D n.m.r. spectrum of the BPTI solution of Fig. 2, which contains numerous α-proton resonances. The chemical shifts at which the cross sections have been taken (Fig. 2) are indicated by the numbers in the upper right corner of each multiplet presentation. The broken lines show the multiplets obtained without double irradiation, the solid lines those with spin decoupling at 1.34 ppm (Fig. 2). Multiplets which show spin decoupling effects are identified by a pair of arrows indicating the residual spin coupling.

shows the cross sections of the methyl multiplets in the 2D spectrum of BPTI obtained without double irradiation, and with spin decoupling at 3.95 ppm (Fig. 2), respectively. In Fig. 4 corresponding results can be seen for the α-proton region of the 2D spectrum. Comparison of Fig. 2 with the undecoupled spectra in Figs. 3 and 4 provides an illustration of the dramatically improved

spectral resolution in the 2D spectrum. Particularly striking is the resolution obtained for numerous multiplets in the α-proton region, where in the one-dimensional spectrum extensive overlap of lines prevails also after digital resolution enhancement (6). In contrast to the situation in the one-dimensional spectrum, complete or partial decoupling can unambiguously be observed in these well resolved multiplets of the 2D spectrum, as is discussed in more detail in the following.

Fig. 3 shows that double irradiation at 3.95 ppm affects the doublets 2, 4, 5, 6 and 20. These were previously assigned with one-dimensional techniques to Thr 54, Thr 11, Ala 58, Ala 40 and Thr 32 (10), with the vicinal protons at 3.95, 4.04, 3.99, 4.09 and 4.01 ppm, respectively (6). The observations in Fig. 3 correspond to the expectations from these parameters. Resonance 2 is fully decoupled, and the residual spin couplings increase in the order $5 < 20 < 4 < 6$. None of the other methyls is coupled with a resonance between 3.74 and 4.29 ppm (6).

In Fig. 4, partial decoupling is observed for the multiplets at 3.19, 3.80, 4.07, 4.33 and 4.34 ppm. The decoupling experiment shows that two multiplets are overlapped in the cross section at 4.34 ppm, of which only one is affected by double irradiation at 1.34 ppm. These data show that even in this very crowded spectral region, informative spin decoupling effects can be seen in the cross section presentation of J-resolved 2D n.m.r. spectra.

DISCUSSION: This is to the best of our knowledge the first report on selective spin decoupling in 2D n.m.r. spectra. The paper shows that the practical difficulties which oppose such experiments can be handled even in work with biological macromolecules. For the near future, it is to be expected that first level resonance assignments, i.e. identification of the complete spin systems of individual amino acid residues (1), in small and medium sized proteins can be obtained with these techniques on a much broader scale than was hitherto possible with one-dimensional double resonance techniques. For the more distant future, one can foresee that extensive first level assignments in proteins could even be achieved on the basis of automatic serial decoupling experiments in 2D spectra.

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