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STUDIES OF J-CONNECTIVITIES AND SELECTIVE ^{1}H-^{1}H OVERHAUSER EFFECTS IN H_2O SOLUTIONS OF BIOLOGICAL MACROMOLECULES BY TWO-DIMENSIONAL NMR EXPERIMENTS

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SUMMARY: This paper demonstrates that two-dimensional (2D) NMR experiments are particularly suitable for studies of biopolymers in H_2O solution. Compared to conventional one-dimensional NMR experiments the 2D studies are much more efficient and yield with a single instrument setting a nearly complete network of J-connectivities or cross relaxation pathways involving labile protons. The use of these techniques in H_2O solutions presents a new approach for detailed studies of the backbone spatial structure in proteins and should further be of particular interest for conformational studies of nucleic acids. Here, homonuclear J-connectivities and Overhauser effects (NOE) involving labile protons were studied in the basic pancreatic trypsin inhibitor.

During the past decade considerable efforts were made to improve the techniques used to record high resolution ^{1}H nuclear magnetic resonance (NMR) spectra of biological macromolecules in H_2O solution (1-4). Interest of biochemists and biophysicists in continued improvements of such experiments arises because of the universal role of aqueous media as physiological environments of biological macromolecules. Furthermore, essential structural information may be lost when H_2O is replaced by $^{2}H_2O$ for ^{1}H NMR studies. This paper presents results obtained with the use of two-dimensional (2D) correlated spectroscopy (5-7) and 2D NOE (8) experiments in H_2O solution of a protein.

In addition to the non-labile hydrogen atoms attached to carbon atoms, proteins and nucleic acids contain labile protons attached mainly to nitrogen and oxygen atoms. In proteins observation of backbone amide protons plays a crucial role in NMR studies of the molecular conformation (3,9-11). While amide protons located in the interior of protein molecules may exchange sufficiently slowly to be observable in $^{2}H_2O$ solution (10), NMR studies in $^{2}H_2O$ are limited to certain regions of the molecular structure of selected proteins and a more

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general application of the technique requires measurements in H_2O. In nucleic acids ^1H NMR studies of secondary and tertiary structure have been based primarily on observations of labile protons in intramolecular hydrogen bonds, which can be observed only in H_2O solution (12,13). It is to be expected that future conformational studies of proteins and nucleic acids will require extensive studies of J-connectivities and cross relaxation networks in H_2O solutions (9-11,14,15). For such investigations the 2D techniques discussed in this paper are much more efficient than conventional 1D experiments and thus promise to greatly enhance the potentialities of high resolution NMR for investigations of biopolymer conformations.

METHODS AND MATERIALS: 2D correlated spectra are obtained with an experimental scheme which includes two non-selective 90° pulses (5):

$$[90^\circ - t_1 - 90^\circ - t_2]_n .$$

During the evolution period between the two pulses, t_1, the various magnetization components are labelled with their characteristic precession frequencies. The second 90° pulse causes transfer of magnetization components among all those transitions which belong to the same J-coupled spin systems. The signal which manifests the ultimate distribution of labelled magnetization components is recorded immediately after the second pulse as a function of the observation time, t_2. The 2D NOE experiment consists of a sequence of three non-selective 90° pulses (7,8),

$$[90^\circ - t_1 - 90^\circ - \tau_m - 90^\circ - t_2]_n .$$

After frequency labelling of the various magnetization components during t_1, cross relaxation leads to exchange of magnetization between nearby protons during the mixing time τ_m. The interval τ_m is kept fixed and the signal recorded immediately after the third pulse as a function of t_2. Both experiments are, in accordance with the general 2D spectroscopy principle (5), repeated for a set of equidistant t_1-values. To obtain an adequate signal-to-noise ratio, n transients are accumulated for each value of t_1. A 2D Fourier transformation of the data matrix $s(t_1,t_2)$ then produces the desired frequency domain spectrum, $S(\omega_1,\omega_2)$.

360 MHz ^1H NMR spectra were recorded on a Bruker HX 360 spectrometer equipped with an Aspect 2000 data system. The previously developed software for handling of the large data matrices obtained in 2D experiments with biological macromolecules (16,17) was used, with slight modifications for the different experiments. The 2D correlated spectrum was recorded using single channel detection, the 2D NOE spectrum using quadrature detection in both dimensions, with the carrier frequency at one end of the spectrum. Transverse components at the beginning of the mixing period and the axial peaks at $\omega_1=0$ were cancelled by addition of groups of 16 experiments with different phases for each value of t_1. These phase cycles will be described in detail elsewhere. The intense resonance of the solvent water was suppressed by selective, continuous time-shared irradiation at the H_2O frequency with a decoupler power of 0.2 W. The "homodecoupler mode" of the Bruker HX 360 spectrometer was employed, i.e. synchronous with the dwell clock the receiver was on for one third and the decoupler for two thirds

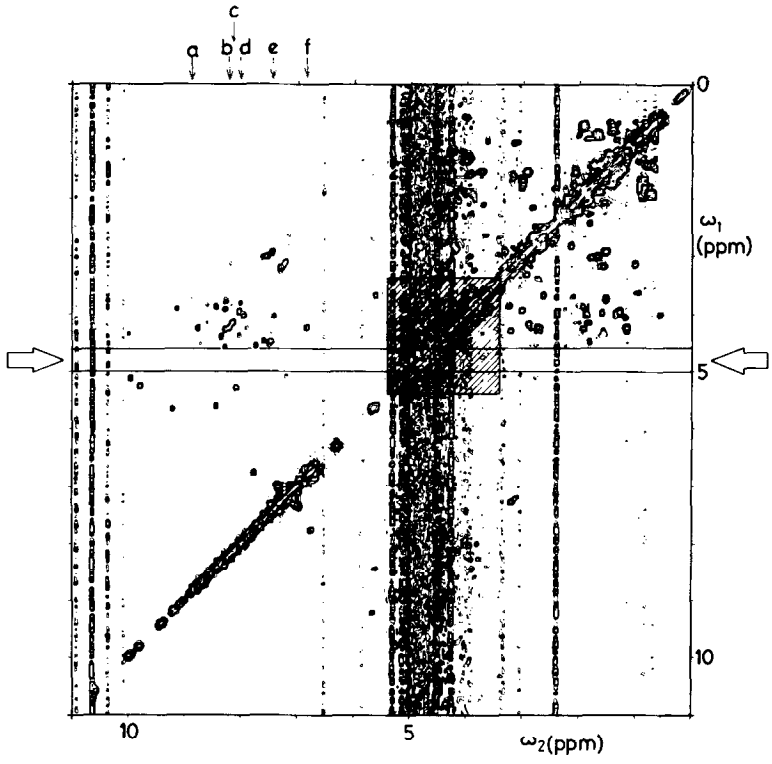


Fig. 1 Contour plot of a 2D correlated ^{1}H NMR spectrum at 360 MHz recorded in a 0.02 M solution of the basic pancreatic trypsin inhibitor (BPTI) in a mixed solvent of 90 % H_2O and 10 % ^2H_2O, pH 4.6, T = 24° C. The spectrometer was locked on the internal ^2H_2O. The spectral width was 4000 Hz. The data set consisted of 512 points in the t_1-dimension and 1024 points in the t_2-dimension. 32 free induction decays were accumulated for each value of t_1, the total accumulation time was ca. 14 h. Before Fourier transformation the free induction decays were multiplied with a phase-shifted sine bell, $\sin[\pi(t+t_o)/t_s]$, where t_s is the experimental acquisition time and t_o/t_s was $\pi/64$ (18). An absolute value plot is shown. The vertical noise bands at 2.45 ppm, between ca. 3.0 and 5.4 ppm, at 6.55 ppm and between 10.0 and 11.0 ppm are discussed in the Methods and Materials section. The horizontal band from 4.60 to 5.00 ppm (area bounded by the arrows on the left and right and the solid lines) indicates the spectral region which was bleached out by the continuous irradiation of the H_2O resonance at 4.80 ppm. The dashed area covers the spectral region which is effectively masked by the vertical noise band near the water line (see text). The arrows labelled a-f at the top of the spectrum indicate where the cross sections of Fig. 3 were taken.

of the dwell time. The intensity of the H_2O line was thus significantly reduced. However in a spectral region of approximately 2 ppm centered about the water line, the individual 1D spectra were quite noisy, which gave rise to a broad vertical noise band in the 2D spectra. In addition there are several quite intense spurious noise peaks running parallel to the ω_1 axis. Further experimental details are given in the figure captions 1 and 2.

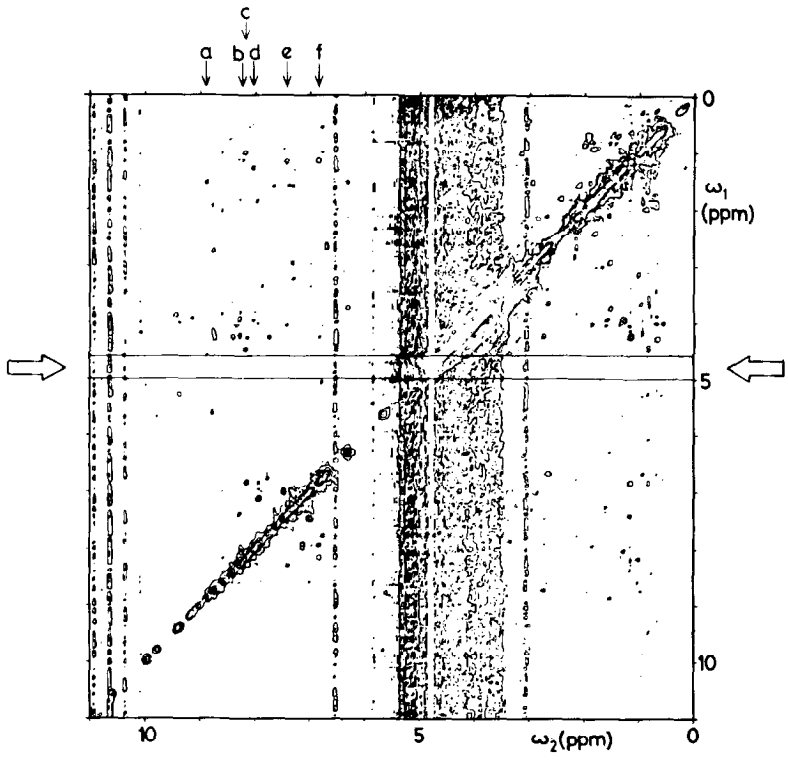


Fig. 2 Contour plot of a 2D NOE spectrum at 360 MHz of the BPTI solution in Fig. 1. The mixing time, τ_m, was 100 msec. Otherwise the experimental parameters and the data handling were the same as in Fig. 1. The spectral region from 4.60 to 5.00 ppm, which was bleached out by the water irradiation, is indicated as in Fig. 1. The arrows a-f at the top of the spectrum indicate the chemical shifts where the cross sections in Fig. 4 were taken.

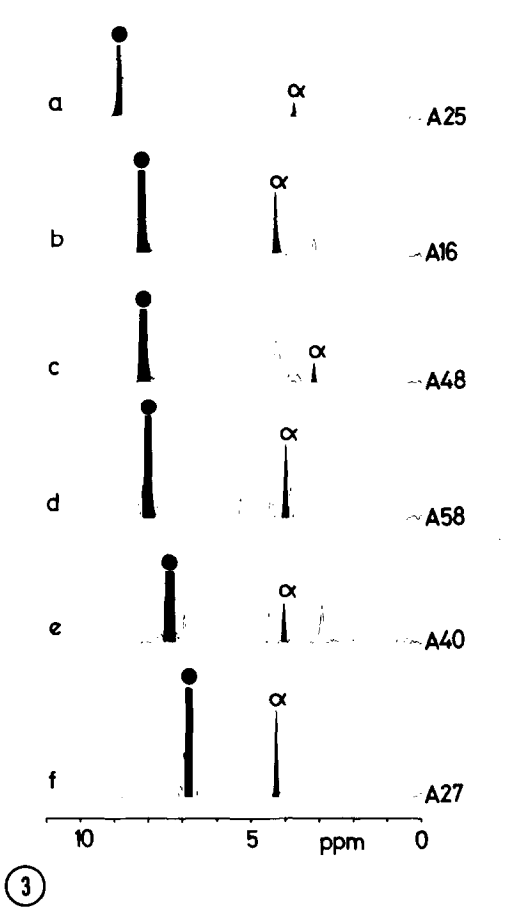
Basic pancreatic trypsin inhibitor, BPTI, (Trasylol®, Bayer Leverkusen) was obtained from the Farbenfabriken Bayer AG.

RESULTS: Figs. 1 and 2 show 2D ^{1}H NMR spectra at 360 MHz of a H_2O solution of the basic pancreatic trypsin inhibitor (BPTI), a small protein of 58 amino acid residues and with a molecular weight of 6500. In both the 2D correlated spectrum (Fig. 1) and the 2D NOE spectrum (Fig. 2) the intense peaks along the diagonal correspond to the normal one-dimensional ^{1}H NMR spectrum of BPTI in H_2O. In the 2D correlated spectrum (Fig. 1) pairs of cross peaks arranged symmetrically with respect to the diagonal manifest J-connectivities between neighboring protons in the covalent structure of the polypeptide chain. In the 2D NOE spectrum recorded with a mixing time of 100 msec (Fig. 2), pairs of cross peaks symmetrical with respect to the

diagonal manifest selective NOE's between groups of protons located at a distance of less than ca. 3.0 Å in the three-dimensional protein structure. In principle, all the proton-proton J-connectivities in BPTI should be contained in the 2D correlated spectrum (5-7) and the 2D NOE spectrum should contain a complete set of NOE's between all closely spaced groups of protons (8). A large number of cross peaks can indeed be distinguished in the spectra of Figs. 1 and 2, and connectivities with the labile amide protons between 6.0 and 10.0 ppm can readily be observed in the upper left triangle of both the 2D correlated and the 2D NOE spectrum in H_2O. Actually, in spite of the incomplete suppression of the H_2O resonance nearly the entire 2D spectra can be analyzed. Not accessible in the spectra of Figs. 1 and 2 are the connectivities with resonances located between 4.60 and 5.00 ppm (outlined in Figs. 1 and 2), since this spectral region is bleached out by the continuous irradiation of the water line at 4.80 ppm. Because of the redundant information content of the two triangles which are separated by the diagonal, essentially only the area containing the correlation peaks between pairs of resonances which are both located in the chemical shift region from ca. 3.4 to 5.4 ppm is masked by the intense vertical noise bands (dashed area in Fig. 1).

Fig. 3 shows the cross sections of the 2D correlated spectrum of Fig. 1 along ω_1 through the diagonal peaks of the amide protons of all six alanyl residues in BPTI. The amide proton resonances were identified from the J-connectivity to the previously identified α-proton lines of the alanines (19). Fig. 4 shows the six cross sections along ω_1 through the diagonal peaks of the alanyl amide protons in the 2D NOE spectrum of BPTI (Fig. 2). NOE's between the amide proton and the α-proton as well as the methyl protons of each alanine are clearly manifested. In addition NOE's between the alanine amide proton and either α-protons or amide protons of the residues preceding and/or following alanine in the amino acid sequence are observed, depending on the secondary structure adopted by the polypeptide segment (9,20).

DISCUSSION: The data in this paper illustrate that 2D NMR is a powerful and efficient method for studies of J-connectivities and selective NOE's in H_2O solutions of biological macromolecules. In spite of the intense solvent line the correlation peaks between resonances in nearly the entire spectral range can be observed, and the two inaccessible regions outlined in Fig. 1 do not really present a serious limitation. Firstly, the spectral region which is



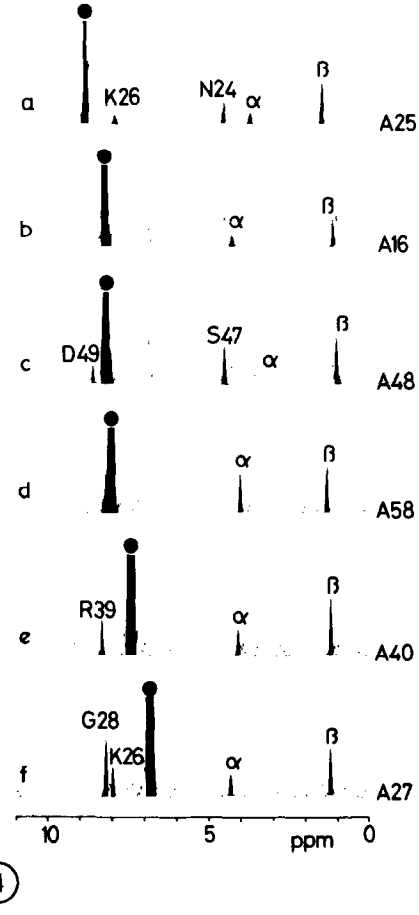


Fig. 3 Cross sections parallel to the ω_1-axis in the 2D correlated 360 MHz ^{1}H NMR spectrum of BPTI in Fig. 1 showing the J-connectivities between the amide protons and α-protons of the six alanyl residues in this protein. The cross sections were taken at the positions of the arrows in Fig. 1, i.e. (a) 8.89 ppm, (b) 8.23 ppm, (c) 8.21 ppm, (d) 8.03 ppm, (e) 7.45 ppm and (f) 6.86 ppm. The truncated diagonal peaks of the amide protons (●) and the α-proton peaks (α) of the alanyl residues indicated on the right of each trace have been shadowed. The additional peaks in the traces b-e correspond to tails of resonances centered in adjoining cross sections.

Fig. 4 Cross sections parallel to the ω_1 axis in the 2D NOE spectrum of BPTI in Fig. 2 taken at the same positions as the cross sections in Fig. 3. The truncated diagonal peaks of the alanyl amide protons (●), the alanyl α-proton (α) and ß-methyl (ß) peaks and identified peaks corresponding to residues preceding or following the alanines in the amino acid sequence have been shadowed. The individual assignments of the alanyl spin systems are indicated on the right of each cross section. In trace a the peaks of the α-proton of Asn-24 and the amide proton of Lys-26 are identified by N24 and K26, respectively. In trace c the amide proton of Asp-49 (D49) and the α-proton of Ser-47 (S47) are observed. In trace e a peak corresponding to the amide proton of Arg-39 is identified by R39, and in trace f the amide proton peaks of Lys-26 and Gly-28 are indicated by K26 and G28. The additional peaks in the traces b, c, e and f correspond to tails of resonances centered in adjoining cross sections.

bleached out by the irradiation of the H_2O line can be sufficiently shifted by temperature variation (3) so that correlations between the labile protons at low field and resonances in the entire spectral region from 0 to 11 ppm can be studied. Secondly, the region from 3.4 to 5.4 ppm does not usually contain resonances of labile protons (3) and hence correlations between different peaks in this range are best studied in a spectrum recorded in ^2H_2O.

To assure that the presentations of the correlation data and the 2D NOE data would be in a directly comparable format, a conventional 2D correlated spectrum (5) was recorded in Fig. 1. As was described in detail elsewhere (6,7) spin echo correlated spectroscopy (SECSY) as well as foldover-corrected correlated spectroscopy (FOCSY) are in many cases superior techniques for studies of J-connectivities in biological macromolecules. In future developments use of the principles of SECSY and/or FOCSY may further improve the potential of 2D techniques for studies of proton-proton J-connectivities and selective NOE's in H_2O solutions of biopolymers.

Further analysis of the correlation peaks in the spectra of Figs. 1 and 2 will be presented elsewhere (20). Besides the identification of the amide proton resonances of the six alanines in BPTI (Figs. 3 and 4), numerous additional resonances could be assigned and new insight into the spatial structure of the protein was obtained (20). The data on the alanines may serve to illustrate what type of information may generally be obtained. As far as the resonance assignments are concerned, the A_3X spin systems of the non-labile alanine protons were previously identified (19) and hence the correlation with the amide protons in Figs. 3 and 4 was straightforward. On the basis of criteria which were previously established when working with very slowly exchanging amide protons in ^2H_2O solution of BPTI (9) the NOE's in Fig. 4 show further that Ala-25 and Ala-48 are in an extended segment of the polypeptide chain, such as the secondary structures in β-sheets, and that Ala-27 and Ala-40 are in "bends" where the amide protons of neighboring residues in the amino acid sequence are in close spatial proximity. Furthermore the relative intensities of the NOE's between the amide proton and the α-proton, and the β-methyl protons, respectively, should provide at least semiquantitative information on the dihedral angle ϕ about the single bond between the α-carbon and the amide nitrogen of the alanyl residues (3).

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