Vol. 113, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 29, 1983 Pages 967-974

APPLICATION OF PHASE SENSITIVE TWO-DIMENSIONAL CORRELATED SPECTROSCOPY (COSY) FOR MEASUREMENTS OF ^1H-^1H SPIN-SPIN COUPLING CONSTANTS IN PROTEINS

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Received May 18, 1983

SUMMARY: Two-dimensional correlated spectroscopy (COSY) is used for measurements of proton-proton spin-spin coupling constants in protein ^1H NMR spectra. High digital resolution along the frequency axis ω_2 is achieved by placing the carrier frequency in the center of the spectrum, using quadrature detection in both dimensions and presenting the spectrum in the phase sensitive mode. Compared to other techniques for studies of spin-spin coupling constants, COSY provides greatly improved spectral resolution. This is illustrated by experiments with H_2O solutions of the small globular protein BUSI IIA (bull seminal inhibitor IIA).

Recently we proposed a general strategy for spatial structure determinations in non-crystalline polypeptides and proteins by high resolution nuclear magnetic resonance (NMR) (1). These procedures rely on sequence-specific assignments for the ^1H NMR lines, which have by now been completed for several small proteins (2-7). The initial steps of the determination of the three-dimensional structure make use of intramolecular distance constraints between distinct groups of protons, which are obtained by nuclear Overhauser enhancement experiments (8-10). Subsequently, a maximum number of additional experimental parameters should be collected and checked for consistency with the protein conformation determined from the ^1H-^1H distance constraints. In this context spin-spin coupling constants between vicinal hydrogen atoms are of particular interest, since the correlations with the molecular conformation have been extensively investigated (11-15). The present paper describes the use of two-dimensional (2D) correlated spectroscopy (COSY) for measurements of spin-spin coupling constants in crowded regions of protein ^1H NMR spectra.

FUNDAMENTAL CONSIDERATIONS: A schematic diagram of a COSY spectrum containing the resonances of two AX spin systems is shown in Fig. 1 (16-18). We distin-

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0006-291X/83 $1.50

guish two types of signals. The "diagonal peaks" are on the diagonal indicated by the broken line and the "cross peaks" lie at positions (ω_1^A, ω_2^X) and (ω_1^X, ω_2^A), where ω^A and ω^X are the chemical shifts of the coupled nuclei. All signals in this example are split into four fine structure components by spin-spin coupling, where the separation of the lines along ω_1 and along ω_2 corresponds to the coupling constant J_{AX}. In a phase-sensitive presentation the cross peaks are in absorption, with alternating sign of the individual fine structure components (Fig. 1), and the diagonal peaks are in dispersion. The presently proposed application of COSY for studies of spin-spin coupling constants in proteins relies on measurement of the separation of fine structure components along ω_2 in the cross peaks.

In small molecules the fine structure of COSY cross peaks has been resolved even in absolute value presentations of the spectra (16,18). However, in absolute value COSY spectra of proteins recorded at high field, which extend over a wide frequency range in both dimensions ω_1 and ω_2 and often contain rather broad lines, the spin-spin coupling fine structure has not usually been exploited. These experiments used relatively low digital resolution (e.g.2-7), both to obtain a workable signal-to-noise ratio with reasonable aquisition times and because of the limited data storage capacities. In the present experiments more efficient use of the storage capacity is obtained by

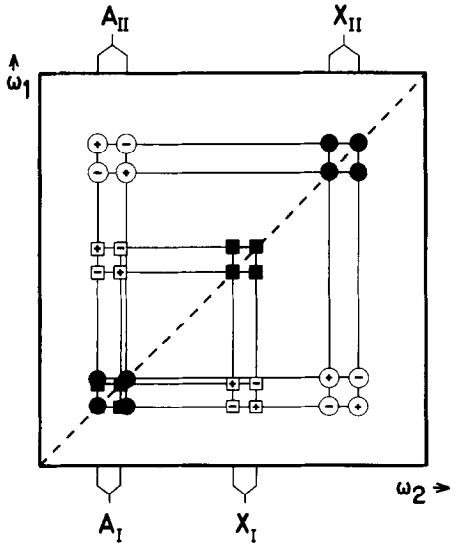
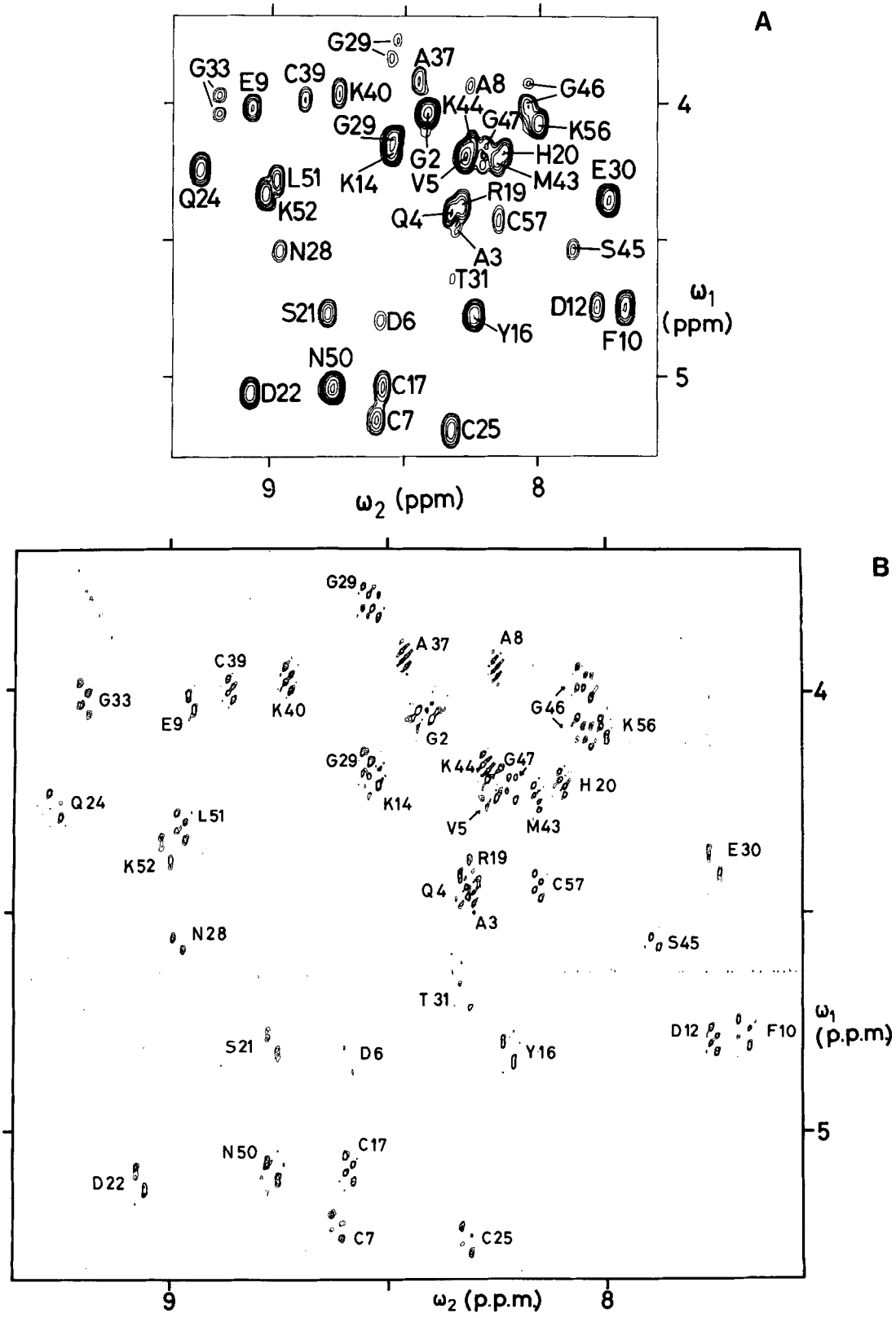


Fig. 1 Schematic diagram of a phase-sensitive COSY spectrum containing two AX spin systems. The diagonal peaks, which have a dispersion shape, are indicated by filled symbols. The cross peaks, which have an absorption line shape and alternate in sign, are indicated by open circles and open squares, respectively, with the sign given by + and -.

placing the carrier frequency in the center of the spectrum, which required a modification of the data accumulation routine. Further it is important that high digital resolution is needed only along one frequency axis (Fig. 1). ω_2 is a natural choice for the high resolution axis both in view of sensitivity of the experiment and of minimal perturbations, for example by the additional irradiation needed for solvent suppression in H_2O solutions (19). Because of the intrinsically broad NMR lines in macromolecules, phase sensitive spectra had to be obtained for better separation of the fine structure components (In absolute value spectra the lines are broadened by the admixture of dispersion mode). Details of the experimental procedures used are given in the last section below.

Compared to conventional 1D NMR and to 2D J-resolved NMR (20-22), which have hitherto been used for studies of spin-spin couplings in proteins (12,13,21,22), COSY can provide greatly improved spectral resolution. This is illustrated by the resonances A_I and A_{II} in Fig. 1. In spite of the near coincidence of the chemical shifts for A_I and A_{II} the cross peaks with X_I and X_{II}, respectively, are well resolved in the ω_1-ω_2 plane. The resonance positions in a 1D spectrum or a 2D J-resolved spectrum can be represented by the diagonal or the non-diagonal fine structure components, respectively, of the diagonal peaks in Fig. 1. The figure shows that the resonances A_I and A_{II} would be overlapped in both of these experiments. In COSY cross peaks such overlap is expected only when both chemical shifts in the two spin systems coincide, which is relatively rare even in the crowded spectra of polypeptides and small proteins.

RESULTS AND DISCUSSION: The following experiments have been recorded with the small globular protein BUSI IIA (bull seminal inhibitor IIA), for which essentially complete sequence-specific resonance assignments are available (7). Fig. 2 shows corresponding regions of a low resolution absolute value COSY spectrum and a high resolution phase sensitive COSY spectrum, which contain the amide proton-C^{α}proton cross peaks of ca. 40 amino acid residues. It is readily apparent that the spectrum 2B contains much sharper peaks and that for each resonance of the low resolution spectrum a fine structure pattern can be observed at the higher resolution. Even though only the positive fine structure components are included in Fig. 2B it is readily apparent that more intricate cross peak patterns appear than in the scheme of Fig. 1. These correspond to the increased complexity of the amino acid spin systems (13) as compared to the AX case. For Cys 57 the NH-C^{α}H cross peak is shown on an enlarged scale (Fig. 3). Along ω_2 only the spin-spin coupling $^3J_{HN\alpha}$ is mani-


A
G29
A37
G33
E9
C39
K40
A8
G46
K44
G47
K56
G29
G2
H20
E30
Q24
L51
K14
V5
M43
K52
R19
Q4
C57
N28
A3
S45
T31
S21
D6
Y16
D12
F10
N50
D22
C17
C7
C25
4
5
9
8
ω2 (ppm)
ω1 (ppm)
B
G29
A37
A8
C39
G33
E9
K40
G46
K56
G2
K44
G47
H20
K14
V5
M43
Q24
L51
K52
R19
E30
Q4
C57
A3
N28
S45
T31
S21
D6
Y16
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ω1 (p.p.m.)

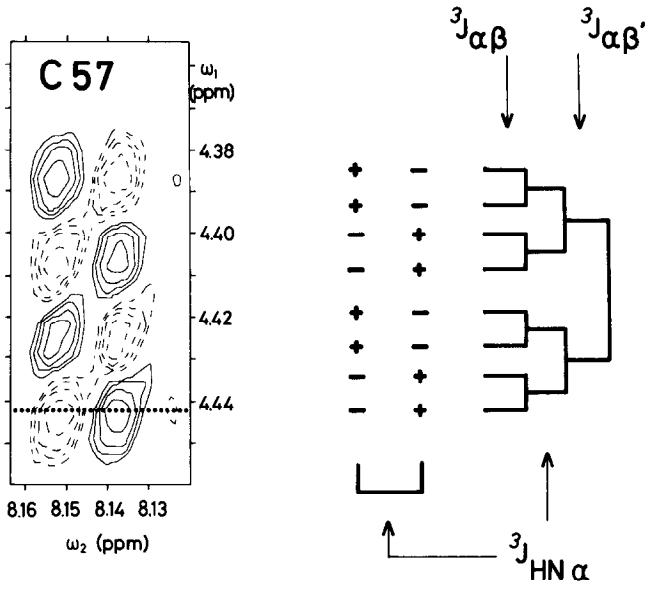

Fig. 3 Analysis of the fine structure of the NH-$C^{\alpha}H$ cross peak of Cys 57 in the phase sensitive COSY spectrum of BUSI IIA in Fig. 2B. On the left this cross peak is shown on an expanded scale. Positive peaks are plotted with solid lines, negative peaks with broken lines. The dotted horizontal line near $\omega_1 = 4.44$ppm indicates where the cross section of Cys 57 in Fig. 4 was taken. The scheme on the right indicates the theoretical fine structure of this cross peak. Along ω_2 only one line separation by the spin-spin coupling constant $^3J_{HN\alpha}$ is expected. Along ω_1 all vicinal coupling constants in the fragment NH-$C^{\alpha}H$-$C^{\beta}H_2$ are manifested, which gives rise to two doublets of doublets (13). Comparison with the experimental spectrum on the left shows that closely spaced fine structure components of equal sign are not resolved, whereas the peak to peak distance between components with different sign is enhanced by the mutual overlap of the absorption lines (see also caption to Fig. 4).

fested, whereas all the vicinal couplings in the fragment -HN-$C^{\alpha}H$-$C^{\beta}H_2$- are represented in the fine structure along ω_1. More details on the spectral analysis are given in Fig. 3 and the caption to this figure. A suitable procedure for measurements of $^3J_{HN\alpha}$ in the direction of the high resolution axis ω_2 is by evaluation of the peak separation in suitably selected (Fig. 3) cross sections (21). As an illustration the cross sections through the NH-$C^{\alpha}H$ cross peaks of all 6 cysteinyl residues in BUSI IIA are shown in Fig. 4.

Fig. 2 Contour plots of the spectral region ($\omega_1 = 3.7$-5.3ppm, $\omega_2 = 7.5$-9.4ppm) of two ^1H COSY spectra recorded in a 0.016M solution of BUSI IIA (bull seminal inhibitor IIA)(23) in a mixed solvent of 90% H_2O and 10% ^2H_2O, pH 4.9, T = 45°C. This spectral region contains most of the amide proton-C^{α}proton cross peaks (7). A. Absolute value spectrum at 500 MHz. The digital resolution was 4.8 Hz/point in both directions. B. Absorption mode spectrum, digital resolution 2.4 Hz/point along ω_1 and 0.4 Hz/point along ω_2. Only the positive fine structure components are displayed.

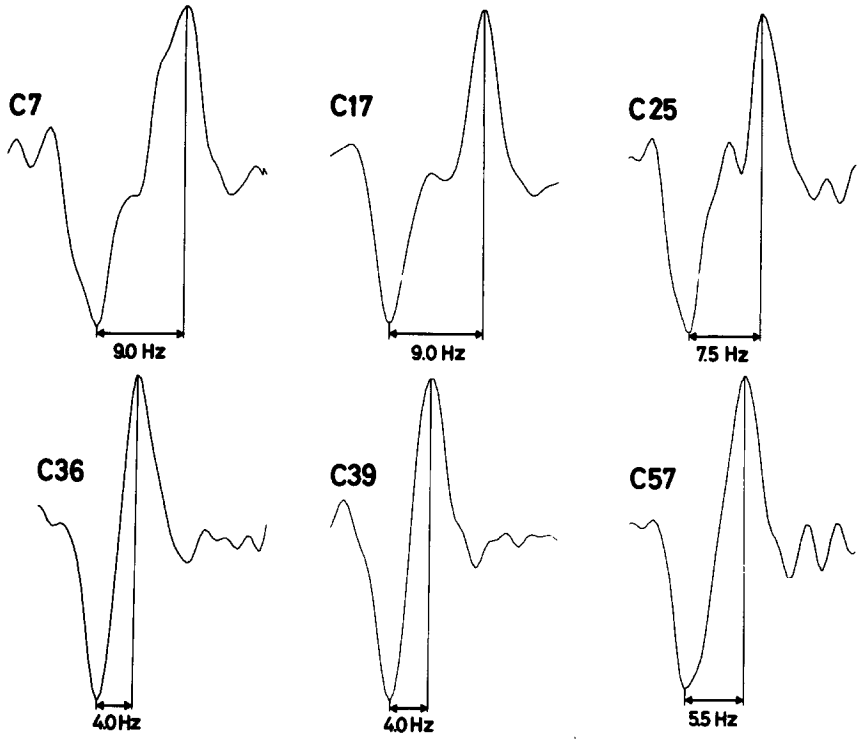

Fig. 4 Cross sections parallel to ω_2 (see Fig. 3) through the NH-$C^{\alpha}H$ cross peaks of the 6 cysteinyl residues of BUSI IIA (23) in the phase sensitive COSY spectrum of Fig. 2B. The separation of the positive and negative peak is indicated for each multiplet. For the larger couplings (upper row) this peak separation corresponds to $^3J_{HN\alpha}$ (Fig. 3). For the smaller couplings (lower row), where the line width is of the same order as the coupling constant, the peak separation represents an upper limit for $^3J_{HN\alpha}$ and a more accurate evaluation of the coupling constants must rely on comparison with spectrum simulations (D.Marion and K.Wüthrich, to be published).

From analysis of the complete NH-$C^{\alpha}H$ region of the COSY spectrum in Fig. 2B the coupling constants $^3J_{HN\alpha}$ were determined for all 57 residues in BUSI IIA except for the prolines and glycines. This data will be presented elsewhere in conjunction with a secondary structure determination in this protein (M. Williamson, D. Marion and K. Wüthrich, in preparation). In principle, similar experiments can be used for measurements of spin-spin couplings for example in the amino acid side chains of proteins and in other macromolecules. However, the spectral analysis may in certain cases be more involved when strong coupling has to be taken into account.

MATERIALS AND METHODS: BUSI IIA was obtained as a gift from Dr. P. Štrop and the NMR sample was prepared as described elsewhere (7).

The phase-sensitive COSY spectrum was recorded on a Bruker HX 360 spectrometer equipped with an Aspect 2000 computer containing a pulser board and a Control

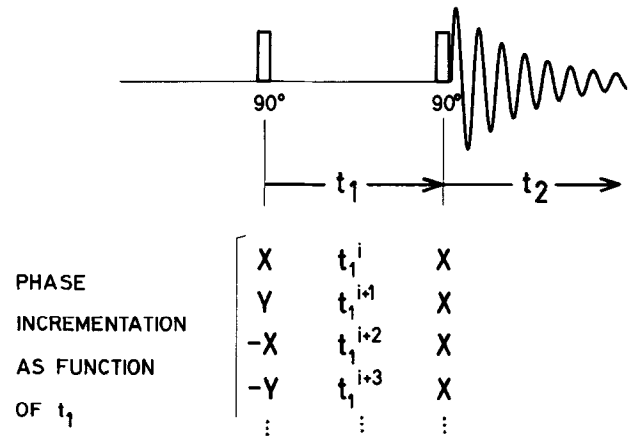

Fig. 5 Experimental scheme used to record COSY spectra of proteins in H_2O solution with four-quadrant phase sensitive display. The basic COSY experiment consists of two 90° pulses, which are separated by the evolution period t_1 (16-18). Immediately after the second pulse the signal is recorded during the observation period t_2. To obtain a 2D spectrum the measurement is repeated for a set of equidistant t_1 values ($t_1^i, t_1^{i+1}, t_1^{i+2}, \ldots$). To end up with a phase sensitive display in four quadrants the phase of the first pulse is incremented by 90° in parallel with each incrementation of t_1, whereas the phase of the second pulse is kept constant. This scheme may be amended with the common routines for suppression of artefacts, for example cancellation of axial peaks (17) and quadrature image suppression (24).

Data Corporation disk drive with a storage capacity of 20 000 kilowords. The pulse sequence of Fig. 5 was used. The phase incrementation of the first pulse amounts to an apparent shift of the rotating frame frequency by $\omega_1/2$ before the analog-to-digital conversion. A comparable two-channel detection has been used to obtain a four-quadrant phase sensitive display of NOESY (2D nuclear Overhauser enhancement spectroscopy) spectra (25). However, the implementation of the experimental scheme of Fig. 5 is quite different in ref. 25 and in the present work, since two spectrometers using a fundamentally different quadrature detection mode were employed. To suppress the resonance of H_2O a continuous irradiation of the solvent line was applied at all times except during t_2 (19). Special care had to be taken in the selection of the carrier frequency near the center of the spectrum, so that the quadrature image of the perturbations arising from the solvent irradiation were outside of the spectral region of interest. 1000 measurements were made, with t_1 values from 1μs to 200ms (The corresponding frequency range along ω_1 of 2400 Hz is less than the total range covered by the spectrum; hence part of the data were folded over). 4096 points were recorded in t_2, with a spectral width along ω_2 of 3600 Hz. To attain the digital resolution indicated in Fig. 2 the time domain data matrix was expanded by "zero filling" to 2048 points in t_1 and 16 384 points in t_2, and to improve the spectral resolution it was multiplied with a phase shifted sine bell in both directions. Because of the limitations on storage space only a part of the data were retained after Fourier transformation in t_2. The spectrum was recorded during ca. 40h, the Fourier transformation for the NH-C^{α}H region required ca. 10h.

The absolute value COSY spectrum of Fig. 2A was recorded on a Bruker WM 500 spectrometer. Quadrature detection was used in ω_2, the carrier frequency was at the low field end of the spectrum. 512 measurements with t_1 values from

0.3 to 51ms were made. To end up with the digital resolution indicated in the Fig. caption 2, the time domain data matrix was expanded by "zero filling" to 2048 points in t_1 and 4096 points in t_2. Similar filter functions were applied as for the phase-sensitive spectrum. The spectrum was recorded in ca. 17h, the Fourier transformation for the entire spectrum required ca. 3h.

ACKNOWLEDGEMENTS: Financial support was obtained from the European Molecular Biology Organisation (EMBO long term fellowship to D. Marion) and the Schweizerischer Nationalfonds (project 3.528.79). We thank Dr. P. Štrop, Institute of Organic Chemistry and Biochemistry CSAV, Prague, CSSR for a gift of BUSI IIA.

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