Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 305-311

# TWO-DIMENSIONAL SPIN ECHO CORRELATED SPECTROSCOPY (SECSY) FOR $^{1}H$ NMR STUDIES OF BIOLOGICAL MACROMOLECULES

K. Nagayama*, K. Wüthrich* and R.R. Ernst**, *Institut für Molekularbiologie und Biophysik and **Laboratorium für Physikalische Chemie, Eidgenössische Technische Hochschule, CH-8093 Zürich, Switzerland

Received July 28,1979

SUMMARY: The principle and the experimental realization of spin echo correlated spectroscopy (SECSY) are described and its use for studies of proteins is illustrated with $^{1}H$ n.m.r. spectra of the basic pancreatic trypsin inhibitor. This technique yields two-dimensional homonuclear correlated spectra which manifest connectivities between spin coupled nuclei and thus provide first level assignments of individual spin systems in biopolymers. Compared to previously described two-dimensional correlated n.m.r. techniques, which were applied exclusively to small molecules, SECSY uses a smaller data size both in the time domain and the frequency domain, which makes it particularly suitable for studies of macromolecules.

One of the fundamental steps in the analysis of complex n.m.r. spectra of biological macromolecules is the identification of the spin systems of individual structural fragments by investigation of connectivities between different components of the spin systems (1). Even though conventional n.m.r. methods include experiments for delineating these connectivities and very high magnetic fields are now available, n.m.r. studies of biopolymer conformation still depend critically on the ability to resolve and assign numerous resonance multiplets in the crowded $^{1}H$ n.m.r. spectra of the macromolecules. Recently we have demonstrated that greatly improved resolution can be obtained in two-dimensional (2D) J-resolved $^{1}H$ n.m.r. spectra of proteins at high field (2,3), and that selective spin decoupling in the 2D J-resolved spectra allows to use the improved spectral resolution for the identification of entire spin systems (4,5). Here we describe how numerous individual spin systems in complex spectra can be identified at once by the application of an experimental scheme which provides homonuclear 2D correlated $^{1}H$ n.m.r. spectra.

It was shown previously that 2D correlated n.m.r. spectra provide a direct manifestation of the connectivities between spin-coupled resonances (6). These techniques have, however, so far been used exclusively for studies of small molecules. Extension for the use with macromolecular systems requires special provisions for handling the large data matrices in the time domain and frequency domain. Spin echo correlated spectroscopy (SECSY) uses a

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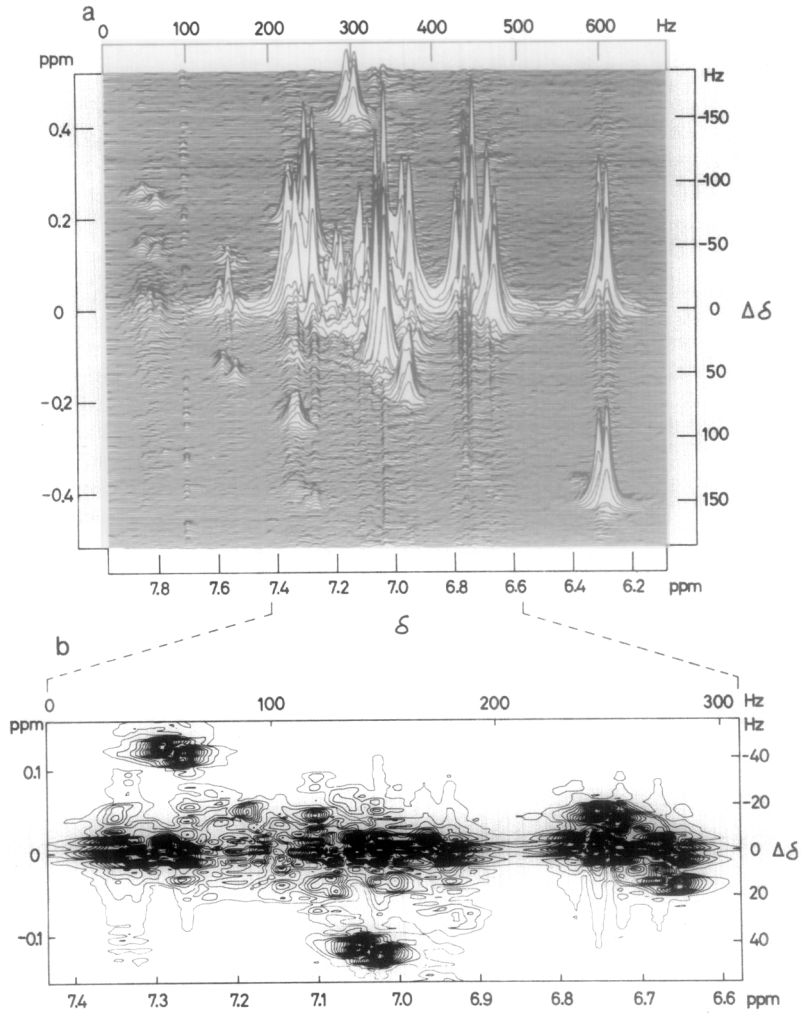


Fig. 1 Aromatic region from 6-8 ppm of the spin echo correlated (SECSY) spectrum recorded in a 0.01 M solution of BPTI in $^2H_2O$, p$^2$H=4.4, T=70° C. (a) Three-dimensional presentation of the entire aromatic region. The chemical shift δ on the horizontal axis corresponds to that in conventional 1-dimensional spectra. Δδ on the vertical axis indicates the difference frequencies of coupled nuclei resulting in a pair of cross peaks at ±Δδ. For convenience both axes have also been calibrated in Hz. (b) Contour plot of the spectral region from 6.6 to 7.4 ppm. In this presentation contour lines connect points of equal signal amplitude.

smaller data size than the previously described experiments (6) and yields a novel presentation of the frequency domain spectra which is more suitable for work with large, complex systems. In the following we first demonstrate the use of SECSY with a protein and describe the analysis of the 2D correlated

spectra thus obtained. The principles of the technique will then be briefly discussed and compared with previously proposed experimental schemes for recording 2D n.m.r. spectra.

APPLICATION TO THE ASSIGNMENT OF SPIN SYSTEMS IN A PROTEIN: Fig. 1 shows the aromatic region of the spin echo correlated $^1$H n.m.r. spectrum of the basic pancreatic trypsin inhibitor (BPTI), a small globular protein with molecular weight 6'500. BPTI contains eight aromatic amino acid residues, i.e. four tyrosines and four phenylalanines, with a total of 36 aromatic protons. The spin systems of the eight aromatic rings were previously identified (7).

To gain access to the information contained in Fig. 1 it may be useful to first consider the schematic SECSY spectrum of an AX spin system in Fig. 2c'. The one-dimensional four-line AX-spectrum is shown at the bottom of Fig. 2c'. A four-line pattern corresponding to the one-dimensional spectrum is contained on the $\omega_1'=0$ line in the 2D spectrum as well. In addition, each of the four component lines appears a second time shifted parallel to the $\omega_1'$-axis by $+\frac{1}{2}J$ or $-\frac{1}{2}J$, so that for each of the two doublets, these two additional peaks define a line which forms an angle of $135^\circ$ with the $\omega_2'$-axis. This results in two four-peak patterns centered on the $\omega_1'=0$ line. Two identical four-peak patterns appear also in positions resulting from shifts of $+\frac{1}{2}\delta_{AX}$ and $-\frac{1}{2}\delta_{AX}$ along the $\omega_1'$-axis. The centers of these two shifted four-peak patterns again lie on a line which forms an angle of $135^\circ$ with the $\omega_2'$-axis. Fig. 2c' shows that a SECSY spectrum manifests simultaneously the chemical shifts, the spin-spin coupling constants and the connectivities between coupled resonances.

For the analysis of Fig. 1 we shall concentrate on the connectivities between coupled resonances. From Fig. 2c' we know that the cross-peaks indicating coupling between two resonances A and X are displaced from the $\Delta\delta=0$ line by $\Delta\delta = \pm\frac{1}{2}(\delta_A-\delta_X)$ (note that $\omega_2'$ and $\omega_1'$ in Fig. 2c' have been substituted by $\delta$ and $\Delta\delta$ in Fig. 1). Inspection of Fig. 1a shows that there is a cross peak pattern at $\delta=6.30$ ppm and $\Delta\delta=152$ Hz and a corresponding pattern at $\delta=7.14$ ppm and $\Delta\delta=-152$ Hz. The two patterns, which lie on a $135^\circ$ line, correspond to the AA'XX' spin system of Tyr-23 (7). Moving from the periphery towards the $\Delta\delta=0$ line we then have a peak at $\Delta\delta=86$ Hz and $\delta=7.34$ ppm and the correlated multiplet at $\Delta\delta=-86$ Hz and $\delta=7.82$ ppm. At $\delta=7.82$ ppm there is also a peak with $\Delta\delta=-40$ Hz which is correlated with a line at $\delta=7.56$ ppm and $\Delta\delta=40$ Hz. These peaks are the AA'MXX' spin system of Phe-45 (7). Here it is particularly striking that the connectivities of the 3,5-ring proton reson-

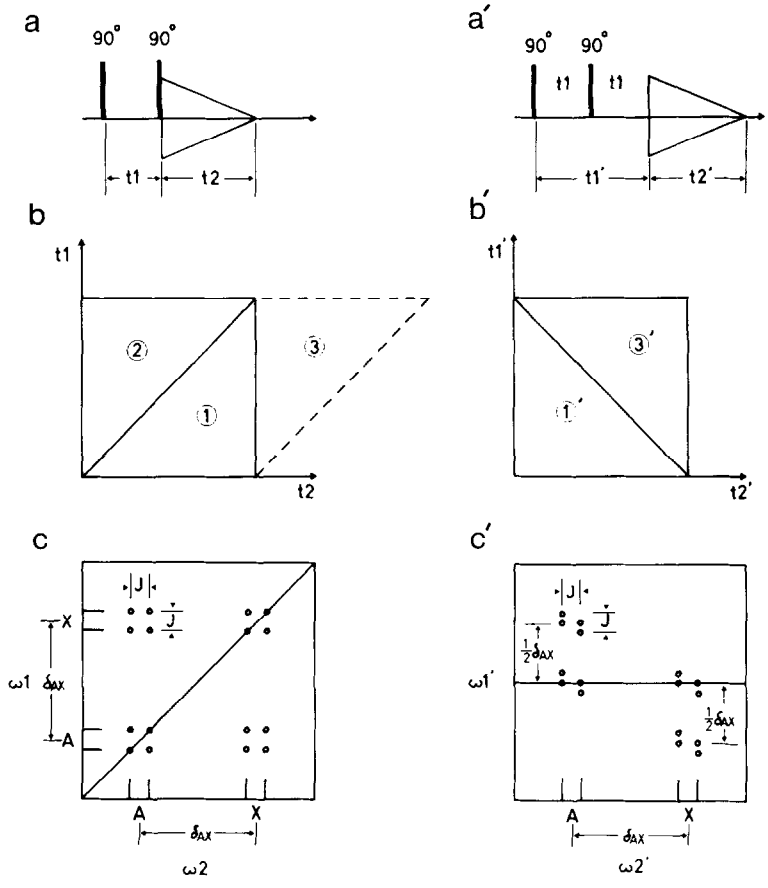


Fig. 2 Comparison of 2D correlated (a,b,c) and 2D spin echo correlated spectroscopy (SECSY) (a',b',c'). The experimental schemes are indicated by the diagrams a and a'. The relations between the recorded data sets $s(t_1,t_2)$ and $s'(t_1',t_2')$ are visualized in the diagrams b and b', where (1) and (1'), and (3) and (3') contain identical data values. The resulting 2D spectra are shown in the diagrams c and c'. They are related by a conformal mapping.

ance at 7.82 ppm with the 4-proton line at 7.56 and the 2,6-proton peak at 7.34 ppm are separately manifested. Next we have a doublet peak at $\Delta\delta$=68 Hz and $\delta$=6.95 ppm which is correlated with a triplet at $\delta$=7.33 ppm and $\Delta\delta$=-68 Hz. These peaks correspond to the two two-proton multiplets of the AA'MM'X spin system of Phe-4.

To analyse the crowded spectral region near the $\Delta\delta$=0 line the contour plot of Fig. 1b provides a more suitable presentation. Two correlated doublets are readily seen at $\delta$=7.04 and 7.28 ppm, with $\Delta\delta$=43 Hz and -43 Hz, respectively. These correspond to the AA'XX' spin system of Tyr-10 (7). Similarly the correlation between the two doublets of the AA'XX' spin system of Tyr-21

(7) at $\delta$=6.67 and 6.74 ppm, with $\Delta\delta$=13 Hz and -13 Hz, respectively, is clearly evidenced.

PRINCIPLES OF 2D SPIN ECHO CORRELATED SPECTROSCOPY: The basic scheme of the experiment is shown in Fig. 2a'. A first 90° pulse creates transverse magnetization which freely precesses for a time $t_1'/2$. Then, a second 90° pulse is applied which initiates the refocusing of the transverse magnetization components. At the peak of the echo at $t=t_1'$, the data acquisition is started, recording the signal $s'(t_1',t_2')$ as a function of $t_2'$. In accordance with the general 2D spectroscopy principle (6), the experiment is repeated for a set of equidistant $t_1'$ values. A two-dimensional Fourier transformation of $s'(t_1',t_2')$, finally, produces the desired frequency domain spectrum.

SECSY forms an intermediate case between the previously described 2D correlated spectroscopy (6) (Fig. 2a) and 2D J-resolved spectroscopy (2,3,8), where the second 90° pulse of Fig. 2a' is replaced by a 180° pulse. We will at first explain SECSY starting from 2D J-resolved spectroscopy and afterwards show the connection with the experiment of Fig. 2a.

Let us consider for simplicity a weakly coupled AX spin system (Fig. 2). The 180° pulse in 2D J-resolved spectroscopy not only induces refocusing but simultaneously interchanges the multiplet components such that the defocusing before and the refocusing after the 180° pulse proceed with frequencies different by the coupling constant J. The apparent precession frequency during $t_1'$ is therefore just $\pm$ J/2, leading to the well-known spread of the multiplet peaks by $\pm$ J/2 in the $\omega_1'$-direction. These peaks are also visible in a SECSY spectrum. Replacing now the 180° pulse by a 90° pulse, there will be complete mixing of all four transverse magnetization components of the AX spin system. At the echo peak at $t=t_1'$, the resulting precession angle corresponds then to an average precession frequency $\omega_1'=\frac{1}{2}(\omega_i-\omega_k)$ where $\omega_i$ and $\omega_k$ are the precession frequencies before and after the 90° pulse, respectively. This leads then to the four $\omega_1'$ frequencies associated with each of the four resonance frequencies along the $\omega_2'$ axis in Fig. 2c'. The possible $\omega_1'$ in an AX system are 0, $\pm$ J/2, $\pm\frac{1}{2}(\delta_A-\delta_X)$ and $\pm[\frac{1}{2}(\delta_A-\delta_X)\pm J/2]$. These frequencies obviously contain both spin coupling and connectivity information as demonstrated in a previous section.

*Fig. 2 demonstrates in addition the close relation between SECSY and the previously described (6) correlated spectroscopy. The two techniques*

differ merely in the time at which acquisition starts (Figs. 2, a and a'), This leads to two related data sets as shown in Figs. 2, b and b', where ① and ①' , and ③ and ③' contain identical data values. Correspondingly, there is also a close relation between the two resulting spectra. Both contain all possible 16 cross peaks and it is possible to convert one spectrum into the other by a simple conformal mapping. However, the major advantage of the SECSY experiment is that it involves, in the $\omega_1$'-domain, exclusively difference frequencies and requires therefore in many cases less $t_1$'-values and consequently less experiments and less data storage than conventional 2D correlated spectroscopy.

When using the simple technique indicated in Fig. 2a', an additional set of peaks appears in the 2D spectrum which form a mirror image spectrum. These undesired peaks can be eliminated by the following modified pulse sequence with phase alternation: $[90^\circ_x - 90^\circ_x - \text{acquisition}(+); 90^\circ_x - 90^\circ_y - \text{acquisition}(-); 90^\circ_x - 90^\circ_y - \text{acquisition}(-); 90^\circ_x - 90^\circ_{-x} - \text{acquisition}(+)]_n$.

EXPERIMENTAL: The basic pancreatic trypsin inhibitor (BPTI, Trasylol®) was obtained from the Farbenfabriken Bayer A.G.. $^1$H n.m.r. spectra were recorded on a Bruker HX-360 spectrometer, using the software which was developed for 2D J-resolved spectroscopy (2,3). The 2D correlated spectrum in Fig. 1 was calculated from a 256x4096 time domain data matrix. The digital resolution is 1.46 Hz on both the $\omega_1$' and $\omega_2$' frequency axes. The accumulation time was approximately 6 hr.

CONCLUSIONS: Fig. 1 demonstrates that 2D spin echo correlated spectroscopy is capable of assigning coupled spin systems even in very complex spectra like those of medium size proteins. From a single SECSY spectrum of BPTI four aromatic spin systems could be completely assigned by inspection. A more careful analysis can reveal many more relations in the coupling network.

2D spin echo correlated spectroscopy can be considered as an alternative to selective decoupling experiments (4,5). It has the advantage to provide all coupling information at once. Many selective decoupling experiments would be necessary to gather an equivalent amount of information. Although spin coupling information is also contained in a SECSY spectrum (Fig. 1c'), due to limited digital resolution, it can usually not be exploited. A complete description of the spin systems in terms of connectivity and accurate spin coupling constants can be obtained if, in addition, a 2D J-resolved spectrum is also recorded. 2D spin echo correlated spectroscopy is of particular advantage when the coupled nuclei exhibit chemical

shifts in the same spectral region, such that the shift differences $\Delta\delta$ cover a limited range only. However, the technique is not limited to this situation and with a sufficiently large data matrix it is also possible to correlate nuclei with widely separated chemical shifts.

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