Vol. 117, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1983 Pages 479-485

IMPROVED SPECTRAL RESOLUTION IN COSY ^{1}H NMR SPECTRA OF PROTEINS VIA DOUBLE QUANTUM FILTERING

M. Rance[+*], O.W. Sørensen[+], G. Bodenhausen[+], G. Wagner[*], R.R. Ernst[+] and K. Wüthrich[*]

[*]Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8093 Zürich, Switzerland

[+]Laboratorium für Physikalische Chemie, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland

Received October 4, 1983

SUMMARY: A double quantum filter is inserted into a two-dimensional correlated (COSY) ^{1}H NMR experiment to obtain phase-sensitive spectra in which both cross peak and diagonal peak multiplets have anti-phase fine structure, and in which the cross peaks and the major contribution to the diagonal peaks have absorption lineshapes in both dimensions. The elimination of the dispersive character of the diagonal peaks in phase-sensitive, double quantum-filtered COSY spectra allows identification of cross peaks lying immediately adjacent to the diagonal, which represents a significant improvement over the conventional COSY experiment.

Two-dimensional correlated spectroscopy (COSY) (1-3) has for some time been employed as an efficient method for the delineation of the spin-spin coupling networks of the individual amino acid residues in proteins (4-6). While the early investigations used absolute value presentations of the data, more recent work showed that phase-sensitive COSY spectra recorded with high digital resolution provide further important advantages (7). For example, with the resolved fine structure of the individual cross peaks unambiguous resonance assignments can be obtained even in crowded spectral regions, and quantitative measurements of the spin-spin coupling constants can be made. A limitation in the use of phase-sensitive presentations of conventional COSY experiments arises because cross peaks and diagonal peaks have different lineshapes (1), which can cause enhancement of the diagonal peaks over the cross peaks in the spectra of macromolecules. As a consequence, important spectral regions near the diagonal are usually not amenable to spectral analysis. The present paper demonstrates the usefulness of applying a double quantum filter (DQF) (8,9) in the COSY experiment of a protein to obtain phase-sensitive spectra in which cross peaks and diagonal peaks have the same lineshapes. The entire spectrum

0006-291X/83 $1.50

thus becomes accessible for detailed analysis and new information on resonance assignments and molecular conformation can be extracted from the DQF-COSY spectra.

FUNDAMENTAL CONSIDERATIONS: A conventional COSY experiment uses the following pulse sequence: 90^{o}-t_1-90^{o}-t_2. The interval t_1 is the evolution period, t_2 the detection period, and the second 90^{o} pulse is usually referred to as the mixing pulse. The forementioned limitations in the use of this experiment arise from the following (10). The diagonal peaks result from that component of the in-phase magnetization which is unaffected by the mixing pulse, so that all multiplet components of a diagonal peak have the same phase and have the binomial intensity ratios. The cross peaks are generated by the transfer of anti-phase magnetization of one spin to anti-phase magnetization of a directly coupled spin; the cross peak multiplets therefore exhibit alternating signs with respect to the coupling which generates the cross peak, and the intensity ratios are no longer binomial. When the cross peaks are phased for pure 2D absorption (i.e. absorption lineshapes in both frequency dimensions), then the diagonal peaks will be in pure 2D dispersion mode. The different 2D lineshapes of the diagonal and cross peaks result from the sinusoidal variation of in-phase to anti-phase magnetization during the evolution and detection periods. The presence of the long dispersion tails makes it difficult to identify cross peaks which lie in the vicinity of the diagonal. Furthermore, since they have alternating signs, the cross peak multiplet components tend to cancel when incompletely resolved. In contrast, no cancellation occurs for the in-phase diagonal peak multiplet components, so that the diagonal peaks in protein COSY spectra typically are enhanced relative to the cross peaks (4-6).

The multiple quantum filter technique (8,9) involves a third 90^{o} pulse immediately following the second 90^{o} pulse of a conventional COSY experiment to convert multiple quantum coherence created by the second pulse back into single quantum coherence. By appropriate phase cycling of the pulses and the receiver it is possible to selectively detect a coherence pathway through a particular order of coherence (11,12). Using a double quantum filter, anti-phase magnetization of one spin is converted by the second pulse into double quantum coherence, which is then immediately reconverted to anti-phase magnetization, either back on the same spin, giving rise to a diagonal peak, or else on the other spin involved in the double quantum coherence, giving rise to a cross peak. Both diagonal and cross peak multiplets then have anti-phase character and, furthermore, can be phased to have simultaneously pure 2D absorption lineshapes. As in conventional multiple quantum spectroscopy (13,14) however, some dispersive anti-phase contributions to the diagonal peaks pass the filter in systems with more than two spins. Fortunately these contributions

are weak and, judged from the results presented below, are tolerable. Because of their anti-phase character in the DQF-COSY spectrum, broad lines do not cause an enhancement of the diagonal peaks relative to the cross peaks, and the latter can be analyzed even when located very close to the diagonal. Earlier work with small molecules also showed that double quantum filtering provides effective suppression of singlet lines (8,9). In the following the practical use of DQF-COSY for studies of proteins is illustrated with experiments on the basic pancreatic trypsin inhibitor (BPTI).

RESULTS AND DISCUSSION: Fig. 1A shows a contour plot of the high field portion of a phase-sensitive COSY spectrum of BPTI in ^2H_2O, and Fig. 1B presents a double quantum-filtered spectrum of the same sample. The lowest contour level plotted for each of the spectra was chosen to be the same fraction of corresponding cross peak intensities. It is obvious that the pure 2D dispersion lineshapes of the diagonal peaks in the conventional COSY spectrum make it almost impossible to observe cross peaks which lie in the vicinity of the

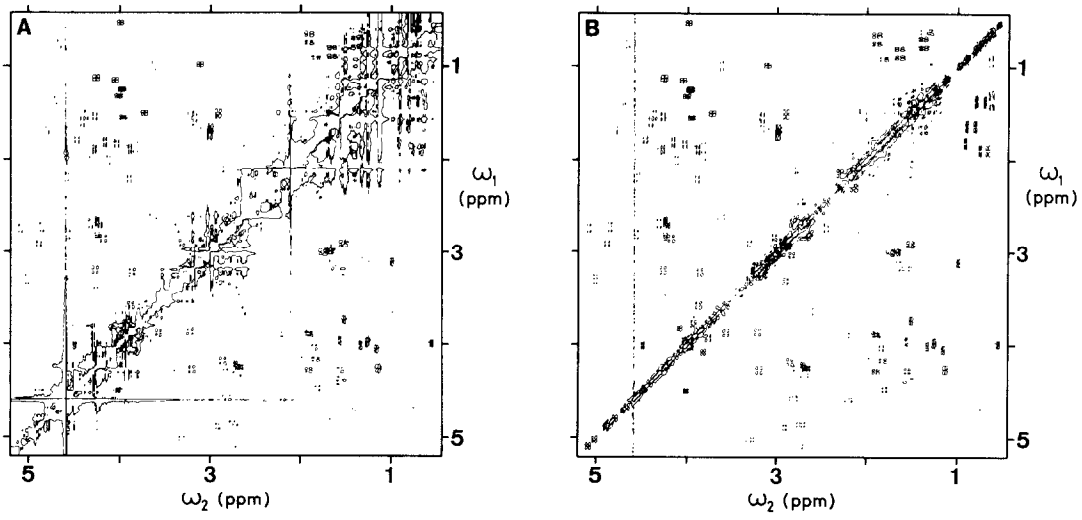

Fig. 1 Comparison of the spectral region from 0.5 to 5.0 ppm of (A) a conventional, phase-sensitive COSY spectrum of basic pancreatic trypsin inhibitor (BPTI) with (B) the corresponding region in a phase-sensitive, double quantum-filtered (DQF) COSY spectrum. The data were recorded at 360 MHz in a 20mM BPTI solution, in ^2H_2O, p^{2}H 4.6, T=36°C. The same filter functions were applied to both data sets, i.e. a phase-shifted sine bell window with phase shifts of $\pi/8$ along t_2 and $\pi/4$ along t_1. The data are presented as contour plots, where both positive and negative levels have been plotted. The COSY spectrum was recorded with 738 t_1 values, from 4 μs to 117 ms, and the DQF-COSY spectrum with 672 t_1 values, from 4 μs to 106 ms. In both spectra 64 transients were averaged for each t_1 value. Note that the singlet resonances of Met 52 at 2.13 ppm and the solvent at 4.65 ppm were suppressed by the double quantum filter, although some of the t_1 noise of the solvent resonance remains.

diagonal. This problem is most pronounced in the spectral region around 1 ppm where the tails of strong methyl peaks interfere with cross peaks.

In Fig. 2A a region of the phase-sensitive COSY spectrum of BPTI, containing the aromatic proton resonances, is plotted on an expanded scale. The interpretation of this part of the spectrum is made difficult by the presence of the long dispersion tails of the diagonal peaks. Fig. 2B shows the results of using optimized window functions (see Materials and Methods) prior to Fourier transformation of the conventional COSY data; while the dispersion tails of the diagonal peaks have been largely suppressed, troublesome distortions of the 2D absorption lineshapes for the cross peaks have been introduced. Although it is possible in principle to eliminate completely dispersion by using a pseudo-echo filter (15), this necessitates the presentation of the data in absolute value mode. Presented in Fig. 2C is the aromatic portion of the DQF-COSY spectrum; this result clearly represents a significant improvement over the conventional COSY spectra shown in Figs. 2A and 2B. The assignments of the aromatic protons in the NMR spectrum of BPTI were made some time ago, with considerable effort, using one-dimensional spin decoupling techniques and spectral simulations (16,17). The DQF-COSY spectrum shown in Fig. 2C allows immediate assignment of almost all of the aromatic protons, and in one instance an earlier assignment must be changed on the basis of the new data. In Fig. 2C the cross peaks of Phe 4 can be unambiguously identified and it follows that the C4H resonance is at 7.31 ppm rather than 7.06 ppm, as concluded from the earlier experiments (17).

As illustrated in Fig. 2C, the double quantum-filtered COSY experiment should be useful in the distinction of cross peaks for the aromatic side chains, such as tyrosine δ-ε couplings, phenylalanine δ-ε and ε-ζ couplings, and tryptophan ε-ζ and ζ-η couplings. Other examples of couplings which give rise to cross peaks that normally are close to the diagonal are glycine α-α, threonine α-β, serine α-β, couplings within methylene groups, and many other couplings within long side chains.

The type of information available from the fine structure of the COSY cross peaks can be illustrated by a few examples from Fig. 2C. The cross peak multiplets for the Phe 4 3,5-2,6 coupling indicate a doublet for the 2,6 protons and a triplet for the 3,5 protons, as expected. The cross peaks for the Phe 22 4-5 coupling indicate triplets for both the 4 and 5 protons. When the distinction of positive and negative intensities is made, the +1:0:-1 intensity ratio characteristic for a cross peak triplet is clearly observed in both frequency dimensions for the Phe 22 4-5 coupling.

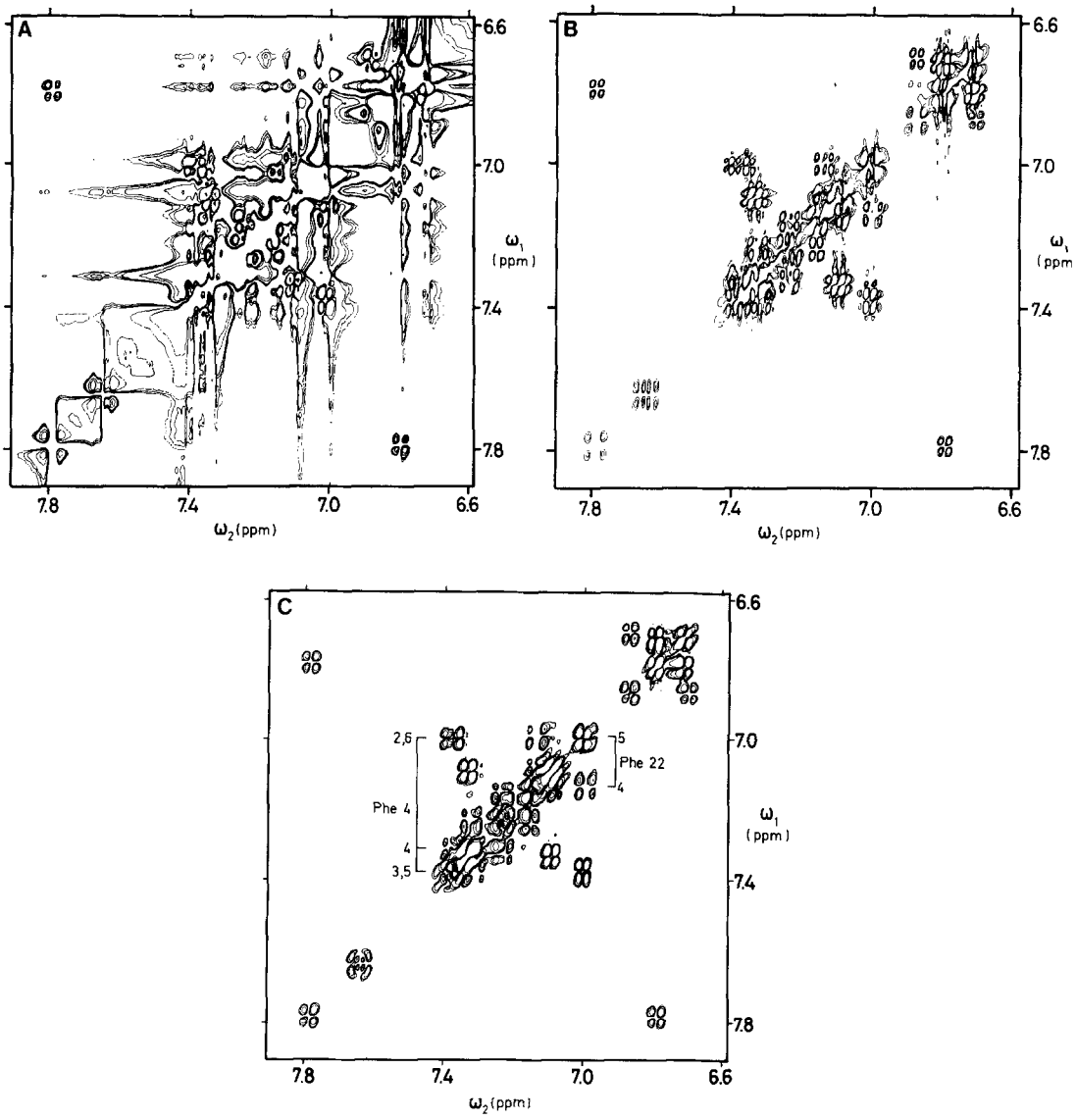

Fig. 2 Comparison of the aromatic region in (A,B) conventional, phase-sensitive COSY spectra of BPTI and (C) a DQF-COSY spectrum. Same data sets as in Fig. 1. Positive and negative levels are shown in the contour plots. The COSY spectrum A and the DQF-COSY spectrum were processed with identical filter functions, i.e. phase-shifted sine bell windows with phase shifts of $\pi/8$ along t_2 and $\pi/4$ along t_1. The COSY spectrum B employed stronger resolution enhancement, with a sine bell-squared window function phase-shifted by $\pi/16$ applied along t_2 and a sine bell phase-shifted by $\pi/32$ along t_1. Note that while the dispersive diagonal has been significantly suppressed, artefacts have been introduced in the cross peak multiplets. In the DQF-COSY spectrum the chemical shifts are indicated for the complete spin system of Phe 4 and for two protons of the non-rotating Phe 22 (17). Note that doublet and triplet fine structures in the cross peaks can be distinquished from the different spacing of the fine structure components: while the spacing for doublets is equal to J, it amounts to 2J for triplets. For example, the cross peak between protons 4 and 5 of Phe 22 has a 1:0:-1 triplet fine structure in both dimensions.

A few comments about the relative sensitivity of the DQF-COSY experiment versus the conventional COSY experiment should be made. In principle, due to the restriction of the coherence transfer through double quantum coherence, the sensitivity of a DQF-COSY experiment is reduced by a factor of two relative to the conventional COSY experiment. In practice, however, the effective sensitivity of a conventional COSY experiment is usually determined not by the signal-to-noise ratio in the normal sense but rather by the amount of t_1 noise and by the size of the cross peaks relative to the intensity of the diagonal peak dispersion tails, as these criteria determine the lowest contour level which can be usefully plotted. Taking these factors into account, the sensitivity of the DQF-COSY experiment is comparable to the conventional COSY experiment.

MATERIALS AND METHODS: The sample used in the experiments was a 20 mM solution of basic pancreatic trypsin inhibitor, BPTI (Trasylol®, obtained as a gift from Farbenfabriken Bayer, Leverkusen), in ^2H_2O, p^{2}H 4.6. The experiments were performed at 36°C on a Bruker AM-360 spectrometer. The conventional COSY spectrum was obtained as described elsewhere (7). The DQF-COSY spectrum was recorded using the following pulse sequence (8,9):

$$(t_o - 90^\circ_x - t_1 - 90^\circ_x - \tau - 90^\circ_\phi - \text{acquisition}(\psi))_{4n}$$

The relaxation delay t_o was 1 s and the interval τ was set to be 4 μs to allow time for switching rf phases between the second and third pulses. The basic four step phase cycling to select for double quantum coherence consisted of cycling the third pulse as ϕ=x,y,-x,-y and cycling the data routing in the ASPECT-2000 computer as ψ=x,-y,-x,y (18). As judged by the degree of suppression of singlet lines, better performance was obtained with further phase cycling by repeating the entire four step cycle four times with the phases of all pulses and the data routing incremented together by 90° each time, giving a sixteen step cycle (18,19). The DQF-COSY spectrum shown in this paper was obtained with the transmitter placed at the low field end of the spectrum, and thus there was no need for quadrature detection in the ω_1 dimension. Quadrature detection in ω_1 can be achieved for a phase-sensitive presentation by incrementing the phase of the first pulse by 90° for successive t_1 values, and processing the data as described elsewhere (7). Alternatively, a second data set can be recorded using the same t_1 values but with the phase of the first pulse advanced by 90°, and the data should then be processed as described by Bachmann et al. (20) and States et al. (21). The data was multiplied by phase-shifted sine bell window functions prior to Fourier transformation in each of the two dimensions.

ACKNOWLEDGEMENTS: We thank Dr. D. Neuhaus for several helpful discussions. O.W.S. acknowledges support from the Danish Natural Science Research Council (J.Nr. 11-3933). This research was supported by the Swiss National Science Foundation (project 3.284-0.82) and by the Kommission zur Förderung der wissenschaftlichen Forschung (project 1120).

REFERENCES:

1. Aue, W.P., Bartholdi, E. and Ernst, R.R. (1976). J.Chem.Phys. 64, 2229-2246.
2. Nagayama, K., Kumar, A., Wüthrich, K. and Ernst, R.R. (1980). J.Magn.Reson. 40, 321-334.

3. Bax, A. and Freeman, R. (1981). J.Magn.Reson. 44, 542-561.
4. Nagayama, K. and Wüthrich, K. (1981). Eur.J.Biochem. 114, 365-374.
5. Wagner, G., Kumar, A. and Wüthrich, K. (1981). Eur.J.Biochem. 114, 375-384.
6. Wider, G., Lee, K.H. and Wüthrich, K. (1982). J.Mol.Biol. 155, 367-388.
7. Marion, D. and Wüthrich, K. (1983). Biochem.Biophys.Res.Commun. 113, 967-974.
8. Piantini, U., Sørensen, O.W. and Ernst, R.R. (1982). J.Am.Chem.Soc. 104, 6800-6801.
9. Shaka, A.J. and Freeman, R. (1983). J.Magn.Reson. 51, 169-173.
10. Sørensen, O.W., Eich, G.W., Levitt, M.H., Bodenhausen, G. and Ernst, R.R. (1983). Prog.NMR Spectros. (in press).
11. Wokaun, A. and Ernst, R.R. (1977). Chem.Phys.Lett. 52,407-412.
12. Bodenhausen, G., Kogler, H. and Ernst, R.R. (1983). J.Magn.Reson. (submitted).
13. Braunschweiler, L., Bodenhausen, G. and Ernst, R.R. (1983). Mol.Phys. 48, 535-560.
14. Sørensen, O.W., Levitt, M.H. and Ernst, R.R. (1983). J.Magn.Reson. 54, (in press).
15. Bax, A., Freeman, R. and Morris, G.A. (1981). J.Magn.Reson. 43, 333-338.
16. Snyder, G.H., Rowan, R.III, Karplus, S. and Sykes, B. (1975). Biochemistry 14, 3765-3777.
17. Wagner, G., DeMarco, A. and Wüthrich, K. (1976). Biophys.Struct.Mechanism 2, 139-158.
18. Bax, A., Freeman, R. and Kempsell, S.P. (1980). J.Am.Chem.Soc. 102, 4849-4851.
19. Hoult, D.I. and Richards, R.E. (1975). Proc.Roy.Soc.Ser.A 344, 311-320.
20. Bachmann, P., Aue, W.P., Müller, L. and Ernst, R.R. (1977). J.Magn.Reson. 28, 29-39.
21. States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982). J.Magn.Reson. 48, 286-292.