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# COMBINED USE OF COSY AND DOUBLE QUANTUM TWO-DIMENSIONAL NMR SPECTROSCOPY FOR ELUCIDATION OF SPIN SYSTEMS IN POLYMYXIN B

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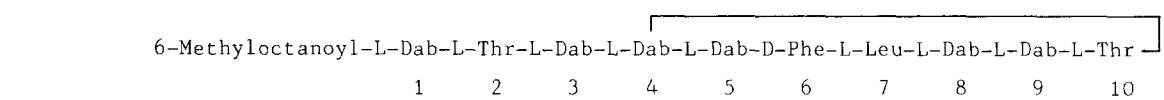
SUMMARY: Two-dimensional single quantum correlation NMR spectroscopy (COSY) and two-dimensional double quantum NMR spectroscopy (2QT) are used to study spin systems in the $^1$H NMR spectrum of polymyxin B. Because of different frequency relationships, the two types of two-dimensional NMR experiments are found to be highly complementary. This is demonstrated by combined use of COSY and 2QT spectroscopy to obtain a complete analysis of the complicated spectral overlap which occurs in the $^1$H NMR spectrum of polymyxin B.

There has recently been considerable activity and interest devoted to determining three-dimensional structures of biological macromolecules in solution by use of two-dimensional (2D) NMR methods [1]. So far these techniques have been very successfully applied to rather small macromolecules, e.g. proteins of molecular weight $\lesssim$ 10,000 [2-4]. For larger macromolecules, the severe spectral overlap encountered even in 2D NMR spectra indicates the need for means of improving spectral resolution. In this communication we show that combined use of 2D single quantum correlation spectroscopy (COSY) [5] and 2D double quantum spectroscopy (2QT) [6] provides complementary information which can be used to unravel extremely complicated cases of spectral overlap. These experiments are applied to polymyxin B, which is a cyclic decapeptide antibiotic with a fatty acid residue joined by an amide linkage to the amino terminus. Cyclization of the peptide involves an amide bond between the γ-amino group of α,γ-diaminobutyric acid 4 (Dab-4) and the carbonyl group of Thr-10.




As shown below, the presence of six Dab residues in polymyxin B leads to severe spectral overlap characteristic of much larger proteins. Polymyxin B therefore serves as an appropriate system for testing the advantages and

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limitations of COSY and 2QT 2D NMR methods for elucidation of amino acid spin systems in proteins.

METHODS: COSY-45 [7] and double quantum two-dimensional NMR spectra were recorded on a Bruker WM-360 spectrometer using 0.04M polymyxin B in $^2H_2O$ at p$^2$H 3.0. Chemical shifts are relative to internal 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionate. For the 2QT spectra, an offset independent multiple quantum transition excitation sequence, now commonly known as INEPT, was used in the accordion manner [6]:

$$90^{\circ}-(\tau_o+\chi t_1)-180^{\circ}-(\tau_o+\chi t_1)-90^{\circ}-t_1-90^{\circ}-\text{Acquisition}(t_2).$$

A 16 step phase rotation cycle, which eliminates zero, first and third order coherences as well as quadrature mismatch along $\omega_2$, was used. Since P and N peaks were not distinguished, the carrier frequency was set at the low field end of the spectrum. The scaling factor $\chi$ was .067 giving a total variation of 5.12 ms for $\tau_o + \chi t_1$. The initial value, $\tau_o$, of 18 ms was selected to optimize double quantum coherence for an AX spin system with a J value of 13.9 Hz. The increment in $t_1$ was chosen so that the sweep width for $\omega_1$ was twice that of $\omega_2$, thereby resulting in a slope of -2 for the main diagonal.

RESULTS AND DISCUSSION: To facilitate comparison of COSY and 2QT NMR spectra, we note that fixed relationships exist between the resonance frequencies observed in these experiments. A COSY spectrum is symmetric about the main diagonal so that off-diagonal cross peaks between directly coupled spins, which contain the information about scalar coupling networks, are located on an antidiagonal (Fig. 1a). When overlap of resonances occurs, e.g. $\omega_C = \omega_D$ in Fig. 1a, COSY spectra cannot be unambiguously interpreted. For example, from the pattern in Fig. 1a it is not clear whether one, both or neither of A and B belong to the same spin system as X.

Such problems with spectral overlap may be overcome by 2QT two-dimensional NMR spectra. 2QT spectra are characterized by lack of diagonal peaks and by cross peaks on lines parallel to the $\omega_2$ axis. For example, in the 2QT spectrum of Fig. 1a, direct coupling between spins with frequencies $\omega_A$ and $\omega_D$ is evident from the presence of a pair of peaks at the common 2QT frequency $\omega_A + \omega_D$. In fact, the frequencies in COSY and 2QT experiments are related by the simple linear transformation $(-\omega_1, \omega_2)_{COSY} \rightarrow (-\omega_1 + \omega_2, \omega_2)_{2QT}$. Hence, the main diagonal in COSY ($-\omega_1 = \omega_2$) corresponds to the main diagonal in the 2QT experiment ($-\omega_1 = 2\omega_2$). Futhermore, a given antidiagonal in the COSY experiment ($-\omega_1 = \omega_1^o - \omega_2$) corresponds to the 2QT frequency ($-\omega_1 = \omega_1^o$) where, for example, $\omega_1^o$ is the 2QT frequency $\omega_A + \omega_D$. From these relationships, it is easily seen that connectivities in the COSY spectrum and the direct connectivities in the 2QT spectrum (solid symbols in Fig. 1a) contain exactly the same information. For detection of these connectivities, the 2QT experiment is only of advantage when the presence of diagonal peaks in the COSY spectrum obscures the off-diagonal peaks, i.e. when chemical shift differences are small (see below). Totally new information is contained

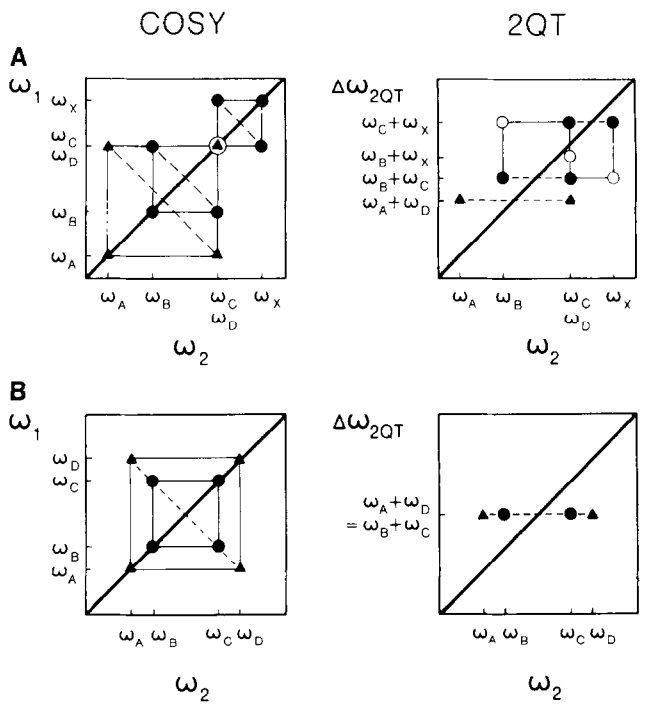


Figure 1. Schematic presentation of frequency relationships in two-dimensional auto-correlated NMR spectra (COSY) and two-dimensional double quantum NMR spectra (2QT). The main diagonals for COSY ($-\omega_1 = \omega_2$) and for 2QT ($-\omega_1 = 2\omega_2$) spectra are shown by heavy solid lines. In the COSY spectra, connectivities between spins of the same spin system are shown by light solid lines and antidiagonals are shown by dashed lines. In the 2QT spectra, connectivities between spins of the same spin sytem are shown by light lines. The dashed portions correspond to the antidiagonals in the COSY spectrum. Direct connectivities are shown by solid symbols and remote connectivities by open symbols. (A) Frequency relationships for (AD) and (BCX) spin systems where $\omega_C = \omega_D$ and $J_{BX} = 0$. (B) Frequency relationships for (AD) and (BC) spin systems where $\omega_A + \omega_D = \omega_B + \omega_C$.

in the remote connectivities evident in a 2QT spectrum (open circles in Fig. 1a). For example, the pattern of remote connectivities in Fig. 1a gives unequivocal evidence that spins B and X belong to the same spin system and A is not part of the system. Finally, we note that despite the added information in a 2QT spectrum, COSY and 2QT spectra are really complementary. This is emphasized in Fig. 1b. Although the two individual spin systems (AD) and (BC) are readily apparent in the COSY spectrum, because all off-diagonal peaks lie along a common anti-diagonal, these spin systems cannot be individually recognized in the 2QT spectrum. Of course, if either the (AD) or (BC) spin systems contain further spins then the two spin systems may be distinguishable at other 2QT frequencies.

Fig. 2 shows the regions of COSY and 2QT NMR spectra which contain the resonances from all six α,γ-diaminobutyric acid (Dab) residues of polymyxin B. The connectivities shown in the COSY spectrum (Fig. 2a) were obtained by

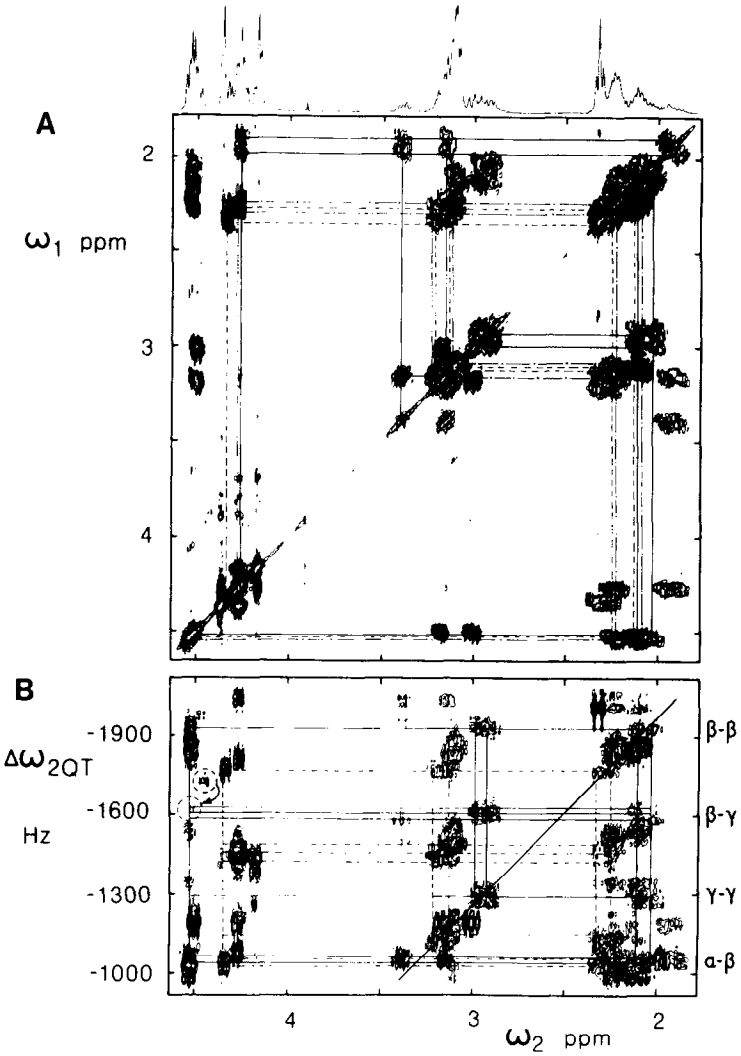


Figure 2. Absolute value contour plots of 2D NMR spectra for polymyxin B showing the spectral regions containing resonances from all six α,γ-diaminobutyric acid (Dab) residues of the polypeptide. (A) COSY-45 2D NMR spectrum. Since the spectrum is symmetric about the main diagonal, connectivities for Dab-1 (———), Dab-2 (– – –) and Dab-3 (—·—·) are indicated in the lower right triangle while connectivities for Dab-4 (———), Dab-5 (—·—·) and Dab-6 (– – –) are indicated in the upper left triangle. The numbering of the spin systems is arbitrary. (B) 2QT 2D NMR spectrum. The main diagonal is indicated by the slanted solid line. Connectivities for Dab-1 (———) and Dab-6 (– – –) are shown. For Dab-1, the hydrogens which show direct connectivities are indicated by the greek letters at the right. The insert shows a four-fold intensity expansion. The frequency scale $\Delta\omega_{2QT}$ corresponds to the offset from the carrier frequency.

combined use of the COSY and 2QT spectra. From the COSY spectrum alone, only the spin system of Dab-4 could be completely unambiguously assigned (Fig. 2a). Although the remaining connectivities in Fig. 2a appear plausible in the COSY spectrum, these assignments were ambiguous because: (1) many of the $\beta CH_2$ and $\gamma CH_2$ groups show degenerate or nearly degenerate resonances for the two

hydrogens of the methylene groups; these patterns were obscured by the diagonal in the COSY spectrum, and (2) many of the different Dab residues show very similar chemical shift patterns; for example, Dab residues 1, 2 and 3 have chemical shifts of 4.523, 4.534 and 4.551 ppm for the αCH hydrogens and chemical shifts of 2.055, 2.108, 2.110, 2.116, 2.233 and 2.249 ppm for the six βCH hydrogens. The αCH-βCH cross peaks for these residues were therefore severely overlapped in the COSY spectrum (Fig. 2a). Similar overlap was observed for the other cross peaks of Dab residues 1, 2, 3, 5 and 6.

Complementary information which allowed complete assignment of the spin systems of the Dab residues was obtained from the 2QT spectrum (Fig. 2b). The 2QT connectivities shown for Dab-1 and Dab-6 illustrate two important points. Firstly, because the 2QT spectrum has no diagonal peaks, the cross peaks near the main diagonal can readily be observed. It is then apparent that the $\beta CH_2$ and $\gamma CH_2$ of Dab-1 as well as the $\beta CH_2$ of Dab-6 all show nearly degenerate resonances for the two hydrogens of the $CH_2$ group. Secondly, because remote connectivities can be observed, there are connectivities which are well-resolved in the 2QT spectrum even though strong overlap occurs in the COSY spectrum. This is exemplified by the resonances of Dab-1 (Fig. 2b). A remote cross peak at the 2QT frequency for the direct connectivities between βCH and γCH hydrogens ($\Delta\omega_{2QT}$ = 1620 Hz) permits easy identification of the αCH chemical shift (insert Fig. 2b). Because only the αCH-βCH connectivity is contained in the COSY spectrum, and because this connectivity was heavily overlapped with αCH-$\beta CH_2$ connectivities of Dab-2 and Dab-3, this assignment could not be obtained from the COSY spectrum. Similar patterns were observed for Dab-6 and allowed assignment of the $\beta CH_2$ and $\gamma CH_2$ resonances despite strong overlap in the COSY spectrum (Fig. 2).

Even within a single spin system, rather different intensities were observed for the 2QT cross peaks (Fig. 2). Some expected cross peaks, particularly remote cross peaks, were of low intensity or could not be observed (insert Fig. 2b). Furthermore, there is considerable overlap of resonances in the 2QT spectrum. This arises from accidental overlap of resonances along an antidiagonal in the COSY spectrum and is illustrated in Fig. 3. In the COSY spectrum, the αCH-βCH cross peaks of Dab-4 lie on the same antidiagonal as the βCH-βCH cross peaks of the phenylalanine spin system (Fig. 3a). Although the two spin systems are clearly distinguishable in the COSY spectrum, they are completely overlapped along $\Delta\omega_{2QT}$ = 1200 Hz in the 2QT spectrum (Fig. 3b). This type of overlap can be resolved by looking at other 2QT frequencies of the individual spin systems. In the present case, the αCH-βCH connectivities of phenylalanine can be clearly observed in the 2QT spectrum along the frequencies $\Delta\omega_{2QT}$ = 660 and 721 Hz. In general, because the connectivities for a single spin system are repeated at several

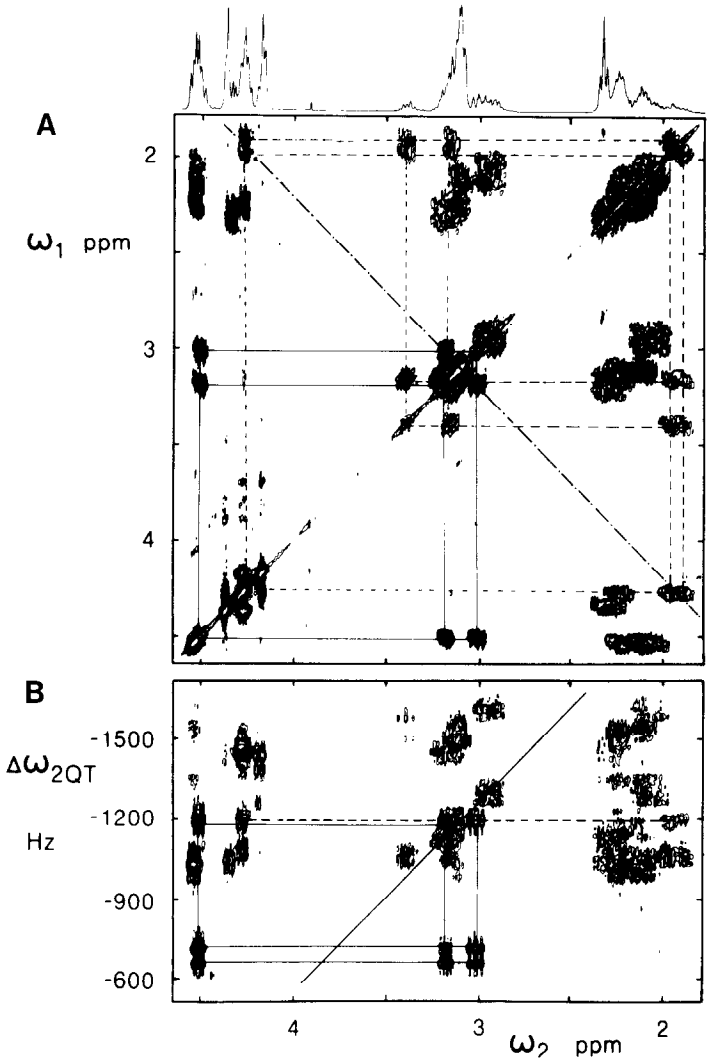


Figure 3. Absolute value contour plots for 2D NMR spectra of polymyxin B showing overlap patterns in COSY and 2QT 2D NMR spectra. (A) COSY-45 2D NMR spectrum. Complete connectivities are shown for Dab-4 (– – – –) and phenylalanine (———). Overlap of the Dab-4 αCH-βCH cross peaks and the Phe βCH-βCH cross peaks along an antidiagonal are indicated by the line (—·—). (B) 2QT 2D NMR spectrum. Connectivities for phenylalanine (———) and a portion of the Dab-4 connectivity (– – – –)are shown. For the 2QT frequency ($\Delta\omega_{2QT}$ = 1200Hz) corresponding to the Dab-4 αCH-βCH and Phe βCH-βCH direct connectivities, the spin systems are completely overlapped.

2QT frequencies and because the COSY spectrum was also available, the intensity variations in the 2QT spectrum and the spectral overlap in both the 2QT and COSY spectra were not serious problems. Complete assignments were obtained for all six Dab residues.

In conclusion, the present results indicate that combined use of COSY and 2QT spectroscopy has great promise for elucidation of spin systems in large biological macromolecules. Although the 2QT spectrum in principle

contains more information, the two methods are best regarded as highly complementary because of the differing patterns of spectral overlap in COSY and 2QT spectra. Indeed, using the principles outlined here, it is possible to predict when combined use of 2QT and COSY spectra will aid in resolution of spectral ambiguities. The present results also emphasize several important aspects of experimental schemes for 2QT spectroscopy. Firstly, although schemes have been proposed for emphasizing direct connectivities in 2QT spectra [8], remote connectivities are more useful for elucidation of spin systems in complicated spectra. Secondly, efficient schemes are needed for excitation of 2QT coherence. In the present case we have used accordion style excitation [6] with this point in mind. Even so, there is considerable variation in the intensity observed for different 2QT cross peaks (Fig. 2b). Hence, thirdly, schemes for efficient transfer from 2QT coherence to transverse magnetization are also needed to assure that remote connectivities are observed. This latter point will be addressed in more detail elsewhere.

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