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A 3D doubly sensitivity enhanced X-filtered TOCSY-TOCSY experiment

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Abstract

We present a 3D double sensitivity enhanced X-filtered homonuclear TOCSY-TOCSY experiment for the assignment of unlabeled molecules complexed to labeled protein- or nucleic acid-domains. The resulting spectrum is clean, can be measured in a reasonable amount of time and allows for increased resolution of overlapping resonances when compared to 2D methods. The 3D X-filtered TOCSY-TOCSY allows for assignment in cases where the size or the composition of the unlabeled molecule results in a high degree of overlap.

Understanding of biochemical processes requires knowledge of interactions between biomolecules, whether they are proteins, nucleic acids or drug molecules. Structural studies of complexes between interacting biomolecules provide valuable information about the structural details of molecular recognition, binding mechanisms, binding specificity and can assist the design of small molecule ligands for proteins or nucleic acids (Zhang and Yuan, 1998; Lo Conte et al., 1999; Nadassy et al., 1999; Dongen et al., 2002). NMR has been established as a technique that can provide such a detailed analysis of these interactions at atomic level (Ikura et al., 1992; Theriault et al., 1993; Omichinski et al., 1993; Billeter et al., 1993; Chuprina et al., 1994; Qin et al., 2001; Pochapsky and Pochapsky, 2001; Zuiderweg, 2002). Typically in these studies, one of the molecules is enriched (labeled) with ^{13}C and/or ^{15}N isotopes, while the other molecule is not. Assignment of the unlabeled molecule is usually performed using the conventional sequential-assignment approach (Wüthrich, 1986), relying on homonuclear 2D X-filtered NOESY and TOCSY spectra (Otting et al., 1986; Ikura and Bax, 1992).

There are cases, however, in which the classical 2D approach falls short, because the spectra show too many overlapping resonances. This problem can be overcome by swapping the isotope labeling of the molecules in the complex. Often, however, this procedure might be too costly or simply not practical, particularly when the ligand is obtainable only by direct isolation from a natural source or cannot be produced effectively by biochemical methods. Here, we report an efficient alternative, the use of a homonuclear 3D double sensitivity enhanced X-filtered TOCSY-TOCSY. We have used this experiment to obtain the complete ^{1}H assignment of an extended fragment of the Sin Interacting Domain (SID) of the protein Mad1 complexed to the PAH2-domain of the protein Sin3B. In contrast to a previous study by Spronk et al. (2000) where a minimal SID-fragment of 13 residues was used, the conventional 2D methods failed on the extended fragment. This extended SID consists of 24 residues: a three residue extension to the N-terminus with respect to the minimal SID and eight additional residues at the more flexible C-terminus, of which 6 residues are either arginines or glutamic acids. This redundancy in amino acid composition resulted in extensive overlap in the 2D X-filtered TOCSY spectrum. However, combining information from the 3D X-filtered TOCSY-TOCSY spectrum and a 2D X-filtered NOESY spectrum resulted in full assignment of the extended SID fragment with exception of the N-terminal residue.

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Table 1. Phase cycling scheme and gradient signs used to generate the four echo-antiecho pairs. The phases ϕ_2 and ϕ_4 refer to the 90°-pulses prior to the two TOCSY mixing periods, cf. Figure 1. The coefficients s_1 and s_2 are used to control the strength of refocusing gradient G3, $G3 = s_1 \cdot G1 + s_2 \cdot G2$. See also the caption to Figure 1

FID	type (t_1,t_2)	ϕ_2	ϕ_4	s_1	s_2
1	PP	+x	−x	+1	+1
2	NP	−x	−x	−1	+1
3	PN	−x	+x	+1	−1
4	NN	+x	+x	−1	−1

Previously, 3D homonuclear TOCSY-TOCSY experiments have been developed for the assignment of unlabeled proteins or oligosaccharides (Cieslar et al., 1990; Rutherford and Homans, 1995). These experiments, however, have not gained much popularity since the relatively low transfer efficiency of a TOCSY experiment results in a low sensitivity. Furthermore, sensitivity is decreased in a regular multi-dimensional TOCSY experiment because the signal resulting from one of the two orthogonal operators $\mathbf{I}_x$ and $\mathbf{I}_y$ is usually lost by the use of a non-isotropic mixing sequence and/or the use of trim pulses or z-filtration in order to remove spectral artifacts. In addition, the number of peaks in a homonuclear experiment increases largely in going from 2D to 3D as a result of the presence of an additional mixing period. (Vuister et al., 1990). In the present study, we used short TOCSY mixing times for both transfer periods, limiting the number of cross peaks. In addition, we included a $^{13}C/^{15}N$ filter to remove signals from the labeled molecule in the complex and we maximized the sensitivity by incorporating sensitivity enhancement schemes for both indirect dimensions.

Both equivalent orthogonal pathways can be retained in a TOCSY-experiment using an isotropic mixing sequence and sensitivity enhancement (SE) (Cavanagh and Rance, 1990; Krishnamurthy, 1995; Sattler et al., 1995; Wijmenga et al., 1996; Köver et al., 1998). In the present implementation, we incorporated a double sensitivity enhancement scheme and thus the 3D experiment retains full sensitivity and is up to twice as sensitive as a regular 3D (Krishnamurthy, 1995; Sattler et al., 1995). Importantly, the insertion of SE in this homonuclear X-filtered experiment did not require additional pulses or delays.

The pulse field gradient coherence selection method (Kay et al., 1992) was used to implement the double sensitivity enhancement in a similar way as previously outlined by Wijmenga et al. (1996) (cf. Figure 1). The magnetization is dephased in both indirect dimensions, t_1 and t_2, by self-compensating gradients G1 and G2, respectively. Since the gradients are inserted in the X-filter delays, there are no additional relaxation losses due to the inclusion of the SE. Gradient G3 is used to refocuss the observable magnetization by the echo-antiecho approach and is combined with the Watergate gradient Go (Piotto et al., 1992). The phases ϕ_2 and ϕ_4 are cycled such to generate PP-, PN-, NP-, or NN-type FIDs. Adding and subtracting of the appropriate FIDs results in a final hypercomplex data set that can be Fourier transformed to a 3D spectrum with in-phase line shapes and improved sensitivity.

TOCSY experiments are not usually run using sensitivity enhancement, since this reinforces phase-twisted line shapes. This effect originates from the transfer of in-phase to anti-phase magnetization, which is inherent for an isotropic mixing sequence. For larger molecules, however, this is less of a problem, because the larger linewidths result in self-cancellation of the anti-phase components. In the present study, the unlabeled molecule of interest is a protein fragment tightly bound ($K_d = 0.28\ \mu M$) to a protein domain in a 1:1 complex of 12.5 kDa. Consequently the linewidths of the peptide signals are as large as those of the protein, resulting in a spectrum virtually free from interfering artifacts. Furthermore, excellent ^{13}C- and ^{15}N-filtering is obtained via three independent ^{13}C-filter delays and two ^{15}N-filter delays. The three ^{13}C-filter delays were set to match the methyl, aliphatic and aromatic $^1J_{CH}$ coupling constant.

Data were acquired at 20.0 °C on a Varian 600 MHz Unity Inova spectrometer equipped with a triple-resonance triple-axis gradient probe. The sample contained 1.3 mM 1:1 complex of unlabeled hMad-SID24 bound to uniformly $^{13}C/^{15}N$ labeled mSin3B-PAH2 at pH 6.3 and was prepared as described before (Spronk et al., 2001). Using 8 scans per FID, $122^* \times 122^*$ echo-antiecho pairs were recorded (∼5 days of measuring time) to sample the complete proton spectrum in both indirect dimensions. Reducing the spectral width in these dimensions can decrease the total measurement time by allowing for folding of the spectrum. The four successive PP, PN, NP and NN-type FIDs were recorded as shown in Table 1 and were combined as outlined by Wijmenga

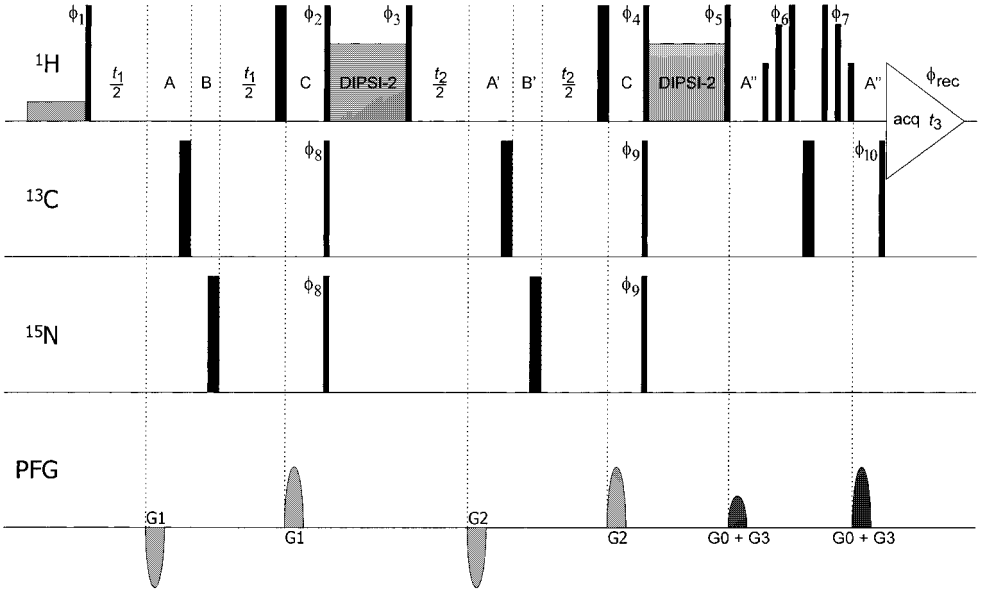

Figure 1. Pulse sequence of the 3D double SE-PFG coherence selection $^{13}C/^{15}N$-filtered TOCSY-TOCSY. Thin and thick bars represent high-power 90°- and 180°-pulses for the proton, carbon and nitrogen-channel at 33.4, 17.1 and 6.6 kHz RF-field strength, respectively. For optimal water suppression we used a 3-9-19 WATERGATE at constant power (Piotto et al., 1992) and low power (38 Hz RF-field strength) presaturation. The filter delays A, A′ and A″ were set to match $1/(4J_{CH})$ for the methyl, the aliphatic and the aromatic proton-carbon couplings (2, 1.79 and 1.5 ms, respectively). The ^{13}C carrier is placed at 40 ppm for filter delays A and A′ and is shifted to 130 ppm prior to delay A″. Delay C is set to $1/(4J_{NH})$ (2.75 ms) and delay B and B′ were set to 0.75 ms and 0.96 ms in order to match the sum of A/A′, B/B′ and C to $1/(2J_{NH})$. The DIPSI-2 sequence was used for the isotropic TOCSY transfer (Shaka et al., 1988). The DIPSI mixing lock axis was set orthogonal to ϕ_2 and ϕ_4 and the mixing time was set to 21 ms for both mixing periods with an RF-field strength of 9.7 kHz. All gradients G*n*(*x*, *y*, *z*) were square-shaped, given along three axis, and were set to the following strengths in Gauss/cm: G1(−7.5, 0, 15); G2(0, −9.3, 18.6); G0(1.5, 0, 0). Gradient duration was set to 0.5 ms for all gradients. G0 is a WATERGATE gradient and is combined with the refocusing gradient G3. The strength of gradient G3 is controlled by the following equation: $G3 = s_1 \cdot G1 + s_2 \cdot G2$, in which s_1 and s_2 are either +1 or −1. PP, NP, PN, NN-type FIDs are obtained by a 180° phase shift on ϕ_2 and inversion of s_1 for every second point in t_1 and a 180° phase shift on ϕ_2 and ϕ_4 and inversion of s_2 for every second point in t_2 (cf. Table 1). All pulses are given along the *x*-axis unless specified otherwise: $\phi_1 = 2(y, -y, x, -x)$; $\phi_2, \phi_3, \phi_5 = 2(x, x, y, y)$; $\phi_4 = 2(-x, -x, -y, -y)$; $\phi_6 = 4(x),4(y)$; $\phi_7 = 4(-x),4(-y)$; $\phi_8 = 4(x), (-x)$; $\phi_9 = 4(x),4(-x)$; $\phi_{10} = 4(x, -x)$; $\phi_{rec} = x, -x, -y, y, -x, x, y, -y$. In the acquisition dimension, the spectral width was set to 9000 Hz and 768 complex points were acquired. In both indirect dimensions, 122* echo-antiecho pairs were acquired sampling a 6000 Hz spectral width.

et al. (1996). The data were processed in straightforward way using NMRPipe (Delaglio et al., 1995).

The increased resolution of the 3D spectrum made it possible to obtain a complete and specific assignment of the 24-residue peptide (N-VRMNIQMLLEAADYLERREREAEH-C). Figure 2 illustrates this. The outer left panel (A) plots the region from the spectrum of the conventional 2D X-filtered TOCSY experiment where contacts are observed between the H^α (F1-axis) and the H^β and H^γ (F2-axis) of four overlapping glutamic acids, E20, E23, E25 and E27. Three of the four glutamic acids can be identified in the 2D spectrum. The H^α frequencies of E23 and E25 can be separated in the 2D, but E20 and E27 have nearly identical chemical shifts for their H^α. The 3D spectrum of the doubly sensitivity enhanced X-filtered TOCSY-TOCSY resolves these two spin systems and also resolves the $H^{\gamma 3}$ of E25 and $H^{\gamma 2}$ of E27. This is shown in the next three panels showing corresponding sections of the (F2, F3)-planes of the 3D spectrum taken at 2.39, 2.35 and 1.93 ppm, corresponding to the $H^{\gamma 2}$ E25/$H^{\gamma 2/\gamma 3}$ E20, $H^{\gamma 2}$ E23 and the $H^{\beta 2/\beta 3}$ E27, respectively. From these sections, it becomes apparent that four spin systems give rise to the crosspeaks, of which the H^α frequencies are indicated by the dashed vertical lines. For instance, the upper peak at (F1, F2, F3 = 2.39, 2.01, 4.10 ppm) in panel B corresponds to the transfer $H^{\gamma 2} \rightarrow H^{\beta 2/\beta 3} \rightarrow H^\alpha$ of E25. The $H^{\gamma 2/\gamma 3}$ of E20 also resonates in this plane of the 3D spectrum, giving rise to a peak corresponding to the transfer $H^{\gamma 2/\gamma 3} \rightarrow H^{\beta 2/\beta 3} \rightarrow H^\alpha$ and the single transfer peak $H^{\gamma 2/\gamma 3} \rightarrow H^{\gamma 2/\gamma 3} \rightarrow H^\alpha$. Likewise, the peaks in panel C correspond to the transfer $H^{\gamma 2} \rightarrow H^{\beta 2/\beta 3} \rightarrow H^\alpha$ and $H^{\gamma 2} \rightarrow H^{\gamma 3} \rightarrow H^\alpha$ of E23 and the lower peak in panel

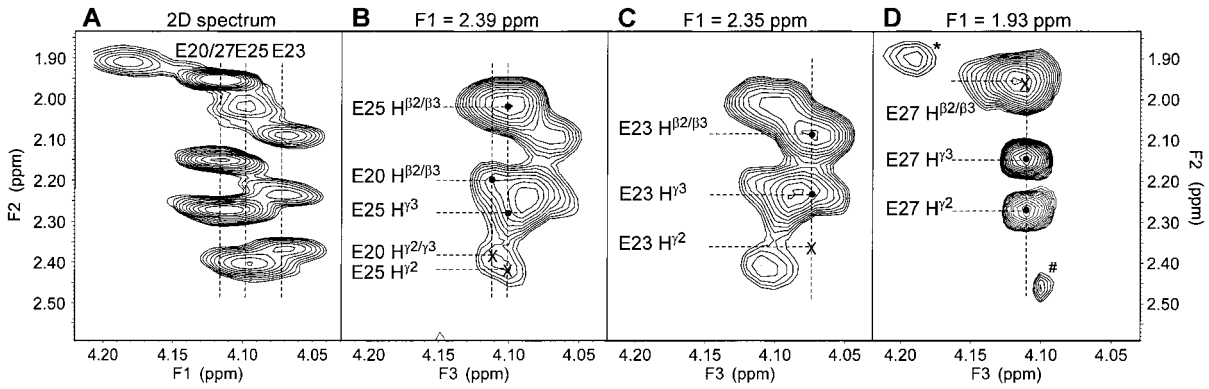

Figure 2. (A) Section from the spectrum of a 2D $^{13}C/^{15}N$-filtered sensitivity enhanced TOCSY experiment showing the contacts between the H^{α} and H^{β} and H^{γ} for the four overlapping glutamic acids E20, E23, E25 and E27 of the SID24-peptide. The three dashed vertical lines indicate the H^{α} frequencies of these residues; the labels refer to their residue numbers. (B), (C) and (D) Sections of (F2, F3)-planes of the 3D $^{13}C/^{15}N$-filtered double SE TOCSY-TOCSY spectrum taken at 2.39 ppm corresponding to the $H^{\gamma 2}$ of E25 and the $H^{\gamma 2/\gamma 3}$ of E20 (B), the successive plane at 2.35 ppm corresponding to the $H^{\gamma 2}$ of E23 (not visible here) (C) and at 1.93 ppm corresponding to the $H^{\beta 2/\beta 3}$ of E27 (D). Again, the vertical dashed lines indicate the H^{α} frequencies. The increased resolution of the 3D spectrum allows for the complete assignment of these four glutamic acids. Peaks on the single-transfer-line (Vuister et al., 1990) are labeled with a cross; genuine 3D-peaks are labeled with a filled circle. The labels indicate the assignment of the crosspeaks in the F2-dimension. The peak labeled with a star corresponds to the transfer $H^{\beta 2} \rightarrow H^{\beta 2} \rightarrow H^{\alpha}$ of R24. The peak labeled with a hash is an artifact. This is easily recognized from its antiphase lineshape in F3 and F1 (not shown). All crosspeaks are non-stereospecifically assigned. Only positive contour levels are plotted.

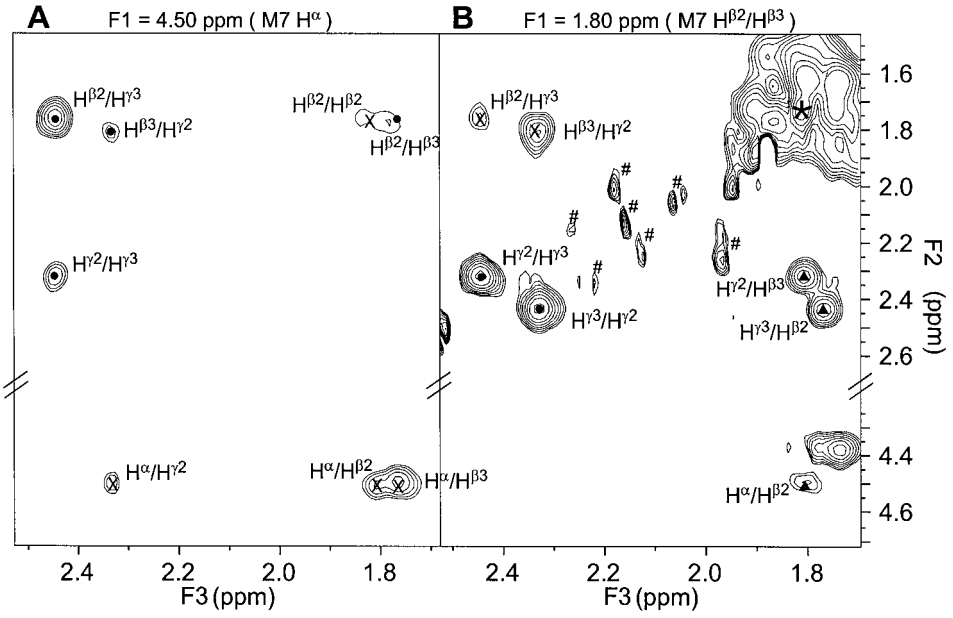

Figure 3. Sections of (F2,F3)-planes of the 3D $^{13}C/^{15}N$-filtered double SE TOCSY-TOCSY spectrum taken at 4.50 ppm corresponding to the H^{α} frequency of M7 (A) and at 1.80 ppm corresponding to the $H^{\beta 2/\beta 3}$ frequency of M7 (B). Again, peaks on the single-transfer-line are labeled with a cross, genuine 3D-peaks are labeled with a filled circle and back-transfer peaks (Vuister et al., 1990) are labeled with a filled triangle. The star indicates the intersection of the 3D body diagonal with the plane. While the region between 1.8 and 2.5 ppm is very crowded in the 2D X-filtered TOCSY spectrum, this is not true for these planes, allowing an easy and complete identification of this spinsystem. The labels indicate the assignment of the crosspeaks in the F2- and F3-dimension. The peaks labeled with a hash result from side lobes in the F1-dimension of intense diagonal peaks.

D corresponds to the transfer $H^{\beta 2/\beta 3} \rightarrow H^{\gamma 2} \rightarrow H^{\alpha}$ of E27.

Another example of the increased resolution of the 3D X-filtered TOCSY-TOCSY is the assignment of M7. The methylene protons of the five glutamic acids, the glutamine, and the two methionines of the extended SID-fragment all resonate in same region from roughly 1.8 to 2.5 ppm. Consequently, crosspeaks between the methylene protons of M7 were obscured in this very crowded region in the 2D X-filtered TOCSY spectrum. This made assignment of M7 impossible, as crosspeaks between the H^{α} and the methylene protons could not be identified as well. Adding a third dimension, however, the crosspeaks between the methylene protons can also be generated starting at the H^{α} of M7. This is shown in panel A of Figure 3, in which peaks corresponding to the transfer $H^{\alpha} \rightarrow H^{\beta 2} \rightarrow H^{\gamma 3}$ and $H^{\alpha} \rightarrow H^{\beta 3} \rightarrow H^{\gamma 2}$ can easily be identified. In a similar fashion, crosspeaks between $H^{\gamma 2}$ and $H^{\gamma 3}$ can easily be identified by starting from the $H^{\beta 3}$ and $H^{\beta 3}$, as is illustrated in panel B of Figure 3. These crosspeaks are unidentifiable in a 2D spectrum, since they are very close to the crowded diagonal.

Using the above approach and a 2D $^{13}C/^{15}N$ double filtered NOESY spectrum (not shown) we obtained the complete sequential assignment of the SID24 peptide bound to the PAH2 domain, which is an essential step in solving the structure of the complex between the two biomolecules.

In conclusion, our 3D double SE X-filtered TOCSY-TOCSY is an effective tool for the assignment of unlabeled molecules with an unusual size or composition. Spectra can be recorded in an acceptable amount of time on millimolar samples of complexes of reasonable size. The third dimension and the inclusion of double sensitivity enhancement extends the limits of the regular X-filtered TOCSY experiment to allow for resonance assignment in cases where extensive overlap prevents the use of conventional two-dimensional methods.

Additional material available

The Varian pulse sequence and parameter file is available on request. The assignments of the extended 24-residue SID-peptide are deposited at the BioMagRes bank under entry number BRMB-6102.

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