Journal of Biomolecular NMR, **15:** 177–180, 1999.
KLUWER/ESCOM

An efficient strategy for assignment of cross-peaks in 3D heteronuclear NOESY experiments

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Received 14 June 1999; Accepted 25 August 1999

Key words: CNH-NOESY, 3D-NOESY, heteronuclear NMR, NCH-NOESY, orthogonal filtering

Abstract

The question is addressed of how maximal structural NOE data on double labelled proteins can be acquired with a minimal set of NOESY experiments. Two 3D-NOESY spectra are reported which, in concert with other commonly used spectra, provide a convenient strategy for NOE assignment. The 3D CNH-NOESY and 3D NCH-NOESY provide NOE connectivities between amide protons and carbon-bound protons and constitute orthogonal heteronuclear filters which eliminate diagonal signals, considerably improving spectral quality. Two different heteronuclear chemical shift dimensions are recorded in the spectra, thus exploiting the extra dispersion of the heteronucleus and considerably simplifying assignment.

Overlap of NOE cross-peaks is still one of the most challenging problems in structure determination by NMR. Heteronuclear edited 4D spectra, preferably employing an optimized folding scheme (Morshauser and Zuiderweg, 1999), are commonly used to resolve overlapping signals. In principle, however, three different 4D experiments, HNNH- (Grzesiek et al., 1995), HCCH- (Clore et al., 1991) and HCNH-NOESY (Muhandiram et al., 1993), are required for complete presentation of all ^{1}H-^{1}H NOEs at their attached heteroatoms. We will show here that almost identical information can be obtained from the derived 3D experiments, NNH-, CCH- and CNH- or NCH-NOESY, which offer the advantages of lower dimensionality while exploiting the commonly larger chemical shift dispersion of the heteronuclei over protons. Whereas the NNH-NOESY has already been described (Zhang and Forman-Kay, 1997), to our knowledge the CNH- and NCH-NOESY experiments have not been reported in the literature.

In most 3D NOESY triple-resonance variants it has been standard to retain two proton dimensions for the nuclei directly correlated by the NOE transfer, and to record the chemical shift of only one attached heteronucleus in the remaining dimension, resulting in spectra with H′,X,H editing. The shift of the other attached heteronucleus in these cases is either recorded in a separate fourth dimension, as in the 4D HCNH-NOESY (Muhandiram et al., 1993), or concommittantly evolves with the first heteronucleus using shared evolution times, resulting in 3D reduced-dimensionality spectra with H′,(C+N),H editing. The major drawbacks of 4D spectra over 3D spectra have been previously described (Bax and Grzesiek, 1993), being mostly the low spectral resolution, the intensity losses due to increased relaxation and the $\sqrt{2}$ intensity loss implicit in the amplitude-modulated complex detection of an additional dimension. This latter $\sqrt{2}$ loss equally holds for 3D reduced-dimensionality spectra which contain pseudo-4D information, i.e. those which evolve the different heteronuclear shifts before and after the NOE mixing (Brutscher et al., 1995; Jerala and Rule, 1995). Employing a phase-modulated transfer scheme (Palmer et al., 1991) can only partially recuperate the lost magnetisation as it invariably lengthens the pulse sequence and thus emphasises relaxation losses.

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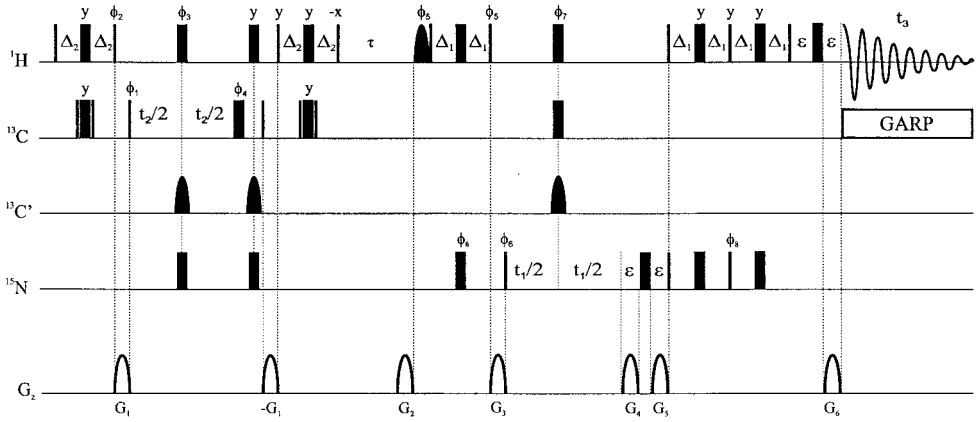

Figure 1. Pulse sequence of the CNH-NOESY. Narrow and wide pulses have flip angles of 90° and 180°, respectively, and are applied along the x-axis unless stated otherwise. The transfer delays Δ_1 and Δ_2 were set to 2.2 ms and 1.3 ms, respectively and the NOE mixing time τ was 80 ms. The water-selective 90° proton pulse with Q5 shape (Emsley and Bodenhausen, 1992) had a duration of 3 ms, while the carbonyl selective 180° inversion pulses were applied with SEDUCE-1 shapes (McCoy and Mueller, 1993) and 300 μs duration. 90° pulses of 90 μs (^{13}C) and 180 μs (^{15}N) were used in GARP decoupling during acquisition. In F1, coherence selection via gradients G_4, G_5 and G_6 was achieved in a phase-sensitive manner by recording echo- and antiecho components separately. Quadrature detection in F2 was achieved via States cycling of ϕ_1. The phase cycles employed were: ϕ_1 = x,−x; ϕ_2 = 8y, 8(−y); ϕ_3 = 4y,4(−y); ϕ_4 = 2x,2y,2(−x),2(−y); ϕ_5 = 16y, 16(−y); ϕ_6 = 4x,4(−x); ϕ_7 = 2x,2(−x); and ϕ_8 = y was inverted along with G_6 for sensitivity enhancement. The receiver phase was ϕ_{rec} = x,2(−x),x,−x,2x,2(−x),2x,−x,x,2(−x),x,−x,2x,−x,x,2(−x),2x, 2(−x),x,−x,2x,−x. The *relative* strengths of the half-sine gradients with equal duration (800 μs), applied at the magic angle with $G_x = G_y = G_z$ for optimal water suppression (Mattiello et al., 1996), were as follows: $G_1 = -20$; $G_2 = 80$; $G_3 = 40$; $G_4 = -60$; $G_5 = 40$; $G_6 = 10.1$ and -10.1 for recording echo- and anti-echo paths, respectively. After each gradient pulse, a field recovery delay of 200 μs was appended, resulting in delay $\varepsilon = 1.0$ ms.

An alternative approach to acquiring 4D spectra is the retention of both heteronuclear chemical shifts in 3D spectra, i.e. X,X′,H editing. In the 3D NNH-NOESY (Zhang and Forman-Kay, 1997) and its analogue, the 3D CCH-NOESY, the second proton dimension is substituted by the heteronucleus, profiting from its better dispersion. Both experiments essentially perform isotope filtering on both protons correlated by the NOE transfer, but as the filters are identical, diagonal signals with X = X′ may pass. However, if the isotope filters are *orthogonal*, i.e. X ≠ X′, spectra which completely lack diagonal signals can be obtained. The lack of strong diagonal signals is highly desirable as the effects of random intensity modulations and phase distortions here become much more pronounced than for the weak NOE cross-peaks. Thus, diagonal signals are notorious for causing t_1-noise and displaying ridges due to minor phase errors. They moreover conceal or distort nearby NOE cross-peaks. We describe here two experiments derived from the 4D-HCNH-NOESY (Muhandiram et al., 1993) which employ orthogonal filters, the CNH- and NCH-edited 3D NOESY experiments, providing complementary information on NOESY connectivities between amide protons and carbon-bound protons. These spectra provide several advantages over standard ^{15}N- or ^{13}C-filtered 3D HSQC-NOESY spectra (HXH-NOESY) in protein structure determination.

Figure 1 shows the pulse sequence for the 3D CNH-NOESY which employs basic INEPT modules for coherence transfer. The coherence follows the path

$$H^C \xrightarrow{\pi^1 J_{HC}\Delta} C(t_1) \xrightarrow{\pi^1 J_{HC}\Delta} H^C \xrightarrow{NOE}$$
$$H^N \xrightarrow{\pi^1 J_{HN}\Delta'} N(t_2) \xrightarrow{\pi^1 J_{HN}\Delta'} H^N(t_3)$$

in the 3D CNH-NOESY and is inverted in the complementary 3D NCH-NOESY. The ReINEPT transfer prior to acquisition is sensitivity-enhanced (Palmer et al., 1991). Water flip-back is achieved through careful choice of proton pulse phases and the suppression of radiation damping through the gradient echo pair G_1 in the first H,X transfer module. An additional water selective 90° pulse is required preceding the second H,X transfer module in order to ensure water flip-back there. Residual water coherence is efficiently dephased by the coherence selection gradients (G_4, G_5 and G_6).

The solution structure of the 185 amino-acid N-terminal domain of VAT, an ATP-ase of the AAA-protein family, has recently been completed in our laboratories (Coles et al., 1999). The two orthogonally filtered NOESY spectra described here were employed extensively during the structure determination. Figure 2 shows a strip from the 3D CNH-NOESY as-

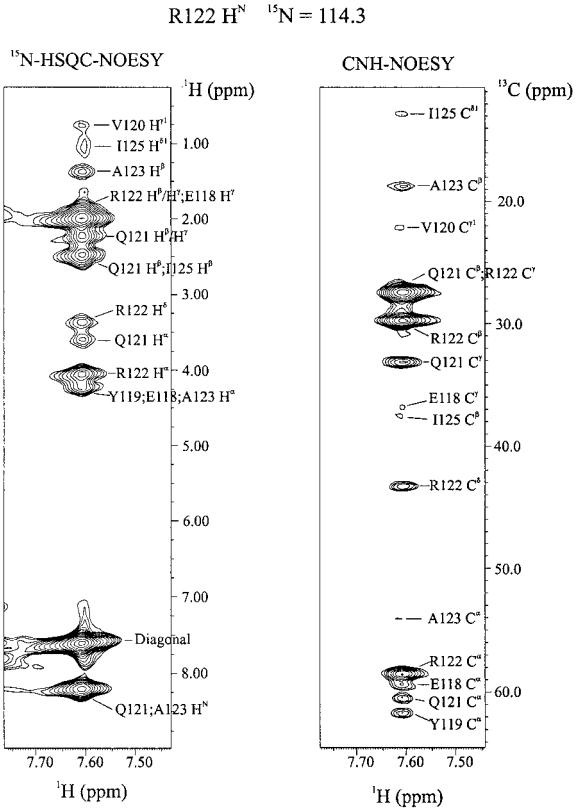

Figure 2. A strip from the CNH-NOESY spectrum assigned to R122-H^N. The greater dispersion of ^{13}C over proton is clearly shown, especially for C^α/H^α where several assignments are available from the CNH-NOESY which were unavailable due to overlap in the ^{15}N-NOESY. Spectra were acquired at 750 MHz, 320 K on a sample of 1.0 mM protein concentration at pH 6.0. The acquired data matrix was 78 × 160 × 1024 complex points with 32 transients. The sweep widths in ^{15}N and ^{13}C were 26.0 and 64.7 ppm, yielding FID resolutions of 26.0 and 76.2 Hz/point, respectively. Other parameters were as given for Figure 1.

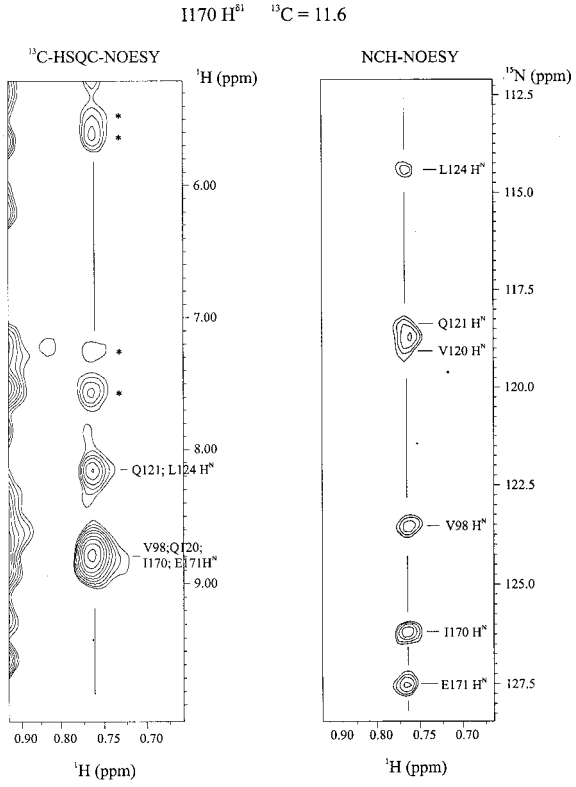

Figure 3. A comparison of the NCH-NOESY with a ^{13}C-HSQC-NOESY, both acquired under the same conditions given in Figure 2. Cross-peaks to amide protons on the strip due to the δ-methyl group of I170 are assigned. Six cross-peaks were identified in the NCH-NOESY which were heavily overlapped in the ^{13}C-HSQC-NOESY. Cross-peaks marked with an asterisk in the ^{13}C-HSQC-NOESY spectrum are due to carbon-bound protons and have no equivalents in the NCH-NOESY. The acquired data matrix was 96 × 132 × 1024 complex points with 32 transients. The sweep widths were as in Figure 2, yielding FID resolutions of 15.0 and 127.0 Hz/point for ^{15}N and ^{13}C, respectively.

signed to R122, demonstrating the excellent intensity and dispersion of signals obtained. R122 is one of six amide groups which have their ^{15}N shifts centred on the plane shown. These display a total of 74 NOESY cross-peaks, 67 of which could be assigned unambiguously and used as distance restraints. In contrast, only 47 cross-peaks to carbon-bound protons could be assigned from the same plane in an HNH-NOESY experiment due to ambiguity and overlap. The advantages of the greater dispersion of ^{13}C over ^1H are particularly apparent for the H^α signals. Four sequential and medium-range contacts involving H^α protons which define an α-helix are clearly resolved in the CNH-NOESY whereas they are hidden by overlap in the HNH-NOESY. Another significant advantage of the CNH-NOESY in the assignment of cross-peaks to H^α protons is the lack of any water exchange cross-peaks for the amide protons which often cause overlap problems for H^α protons with shifts close to that of water.

The orthogonally filtered spectra also provide advantages for the assignment of other aliphatic protons and especially for methyl groups. This is partly due to the much better dispersion of ^{13}C over ^1H shifts for methyl groups and partly to the benefits of the lack of strong diagonal signals. Figure 3 shows a detail of the NCH-NOESY in comparison to a ^{13}C-HSQC-NOESY. The strip pictured is assigned to the δ-methyl group of I170 which shows contacts to six amide protons. None of these assignments were available

unambiguously from the ^{13}C-HSQC-NOESY. I170 lies at the edge of a β-sheet adjacent to the α-helix which includes R122. The extra long-range contacts available from the NCH-NOESY were important for establishing the orientation of the sheet and helix in the calculated structure.

One disadvantage of orthogonal filtering must be mentioned, i.e., is the complications involved in accurately scaling NOE intensities. Two heteronuclear transfers are inherently involved in the technique and cross-peak intensities will be affected by the efficiency of each transfer. Thus, even cross-peaks on the same strip are not directly comparable. This problem, however, is common to all HSQC-NOESY-HSQC-type spectra (and their HMQC analogues, respectively) and can generally be solved by scaling according to the respective 2D-H,X correlation spectra.

It is readily apparent that the CNH- and NCH-NOESY spectra are complementary, i.e. each peak in one spectrum should have a diagonally related partner in the other. Although the two spectra hence contain redundant information, their complementarity provides a means of verifying assignments. It also compensates for the major disadvantage of the CNH-NOESY spectrum, i.e. the loss of diastereotopic information for CH_2 groups, which can easily be retrieved by reference to the NCH-NOESY. In combination, the spectra provide pseudo-4D information while maintaining the superior resolution and signal-to-noise ratio of 3D spectra.

The approach of combining 3D spectra to provide pseudo-4D information has been applied as a general strategy for NOESY assignment in our laboratories. Cross-peak assignments are confirmed by reference to their diagonally related partners, or to corresponding peaks in other 3D spectra, such as ^{15}N- and ^{13}C-HSQC-NOESY spectra. We have found the most powerful combination to be that between the orthogonally filtered NOESY spectra described here and the 3D NNH- and 3D CCH-NOESY spectra. This combination provides both proton and heteronuclear shifts, i.e. pseudo-4D information, for all NOEs; in fact, the same information as is available in three 4D spectra. This set of optimally dispersed 3D-NOESY experiments could be further reduced to three by omitting the CNH-NOESY. The reduction in experimental time, however, results in the loss of pseudo-4D information for the NOEs between nitrogen- and carbon-bound protons, such that some overlapped peaks might remain undetected. The complementary NCH-NOESY should not be omitted, as it allows for diastereotopic discrimination.

In conclusion, we have found a strategy based on the combination of 3D heteronuclear NOESY spectra to be both powerful and easy to apply in the assignment of NOE cross-peaks. In particular, the two spectra described here provide many assignments not available from standard ^{15}N- and ^{13}C-HSQC-NOESY spectra. The ready availability of large numbers of unambiguous NOEs greatly speeds up the process of assignment, as it allows the calculation of accurate model structures early in the assignment process. Thus the strategy lends itself to incorporation in automated NOE assignment routines.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

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