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COMMUNICATIONS

Relayed NOE Experiments for Discrimination of Exchange Effects of Overlapping Labile Protons

BAS R. LEEFLANG AND JOHANNES F. G. VLIEGENTHART

Department of Bio-Organic Chemistry, Bijvoet Center, Utrecht University, P.O. Box 80.075, NL 3508 TB, Utrecht, The Netherlands

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For conformational analysis of biomolecules NMR spectroscopy has become a powerful tool. Especially homonuclear proton 2D NOE spectroscopy (NOESY), giving interproton distances or chemical exchange rates, provides essential information in this respect (*1–3*). Compared to 1D NMR, the suitability of 2D NMR for investigating crowded spectral regions is much greater, due to the creation of cross peaks between two resonances. However, the digital resolution in 2D NMR is limited and resonances frequently overlap. This feature is prominent in the analysis of carbohydrates.

The aim of this study was to retrieve information about intramolecular hydrogen bonding in carbohydrates in aqueous solutions by measuring exchange rates of hydroxyl protons with water protons by means of NOESY experiments. However, when two hydroxyl resonances overlap, only an averaged value for the exchange rate can be obtained, making it impossible to locate a potential hydrogen bond in the molecule. To overcome this problem Wagner (*4*) proposed a relayed NOESY sequence. By adding a relay sequence after the NOE mixing, it is possible to measure the NOE effect between two protons at the resonance frequency of a third proton. A drawback of this method may be low sensitivity due to signal canceling of antiphase magnetization when the coupling constants or the T_2 values are small. Recently, several variants of this technique have been published in 2D NMR (*5–8*) and in 3D NMR (*9–11*). Kessler *et al.* (*5*) proposed the addition of a MLEV isotropic mixing period after the NOE mixing, which has the advantage of a higher sensitivity compared to a relay sequence. A disadvantage of this method may be that magnetization can be transferred along more than one coupling to other spins in the spin system resulting in new overlap.

Here we present an extension of the NOESY–HOHAHA experiment, with a MLEV-17 mixing period. Since presaturation of the water signal (the standard water suppression method) is not applicable when aiming at measuring signals of protons that exchange with water, a semiselective pulse to minimize the excitation of the water signal was incorporated to allow the observation of their signals. This NOESY–HOHAHA experiment with a semi-*s*elective water *s*uppression will be referred to as NOHOSS. In the past few years many articles have appeared about selective excitation techniques (*12–21*). Takegoshi *et al.* give an overview of available sequences (*21*). Although any semiselective pulse can be used, a pulse sequence introduced by von

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Kienlin *et al.* (*20*) was chosen, because this pulse is fast (1 ms) and easy to implement, and it does not induce a large frequency-dependent phase correction nor a severe baseline roll. The last is especially important when there are labile protons resonating close to the water line. They proposed three solvent suppression pulse sequences with different excitation profiles, constructed by combining the jump and return sequence (*12*) with refocusing pulses. From these the following sequence was selected:

$$[\{\text{excitation}\}-\tau-90°]-t-[45°-2\tau-90°-2\tau-45°]\text{Ex}-t-\text{Acq.}, \quad [1]$$

wherein t is a short waiting time to allow hardware switching and τ is a waiting time in the range of 100 to 500 μs to adjust the width of the excitation profile. The refocusing pulses follow an Exorcycle phase program (*22*) denoted by Ex. This semiselective pulse with a frequency response of $\sin^5\omega$ was implemented in the NOESY–HOHAHA sequence. As excitation pulse in Eq. [1] (in (*20*) a simple 90° pulse) the complete NOESY–HOHAHA sequence was used to give the scheme in Fig. 1. The minimal phase program suitable for this sequence is shown in the legend to Fig. 1, and it includes the Exorcycle for the semiselective pulse and suppression of axial peaks. Just after the spin-lock pulse the magnetization is aligned along the axis of the spin-locking field in the xy plane, as it would have been when only a 90° pulse was given. It appeared that the water suppression in one single scan is optimal when the water magnetization does not get inverted after the waiting time τ. Pulse imperfections in the semiselective pulse sequence cause a slight excitation of the inverted water signal, sufficient to start the stimulated emission process, known as radiation damping (*23*). Using TPPI (*24*) with a 90° phase increment only on the first pulse, one of four increments aligns the water magnetization along the negative z axis after the waiting time τ. To avoid dynamic range problems, in every third and fourth experiment the phase ϕ_5 was given a 180° increment to align the water magnetization along the positive z axis. This technique allows observation of signals at more than 350 Hz from the water line, provided that a baseline correction can be performed prior to the Fourier transformation of the t_1 domain.

The pulse sequence described above results in an asymmetric 2D NMR spectrum. The principal cause of asymmetry is the occurrence of two mixing times of different nature. The asymmetry is introduced into the spectrum when magnetization is transferred both through space and through the scalar coupling, giving rise to a cross peak on only one side of the diagonal, as is shown in Fig. 2. Obviously, also symmetric cross peaks are present, stemming from magnetization transfer during only one of the mixing periods. An additional, trivial cause of asymmetry is nonuniform excitation,

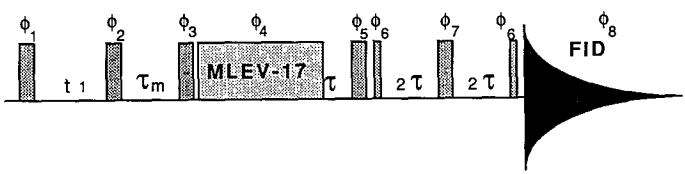

FIG. 1. Pulse sequence of NOESY–HOHAHA with semiselective solvent suppression (NOHOSS). The phase program used was $\phi_1 = 8(x), 8(-x)$; $\phi_2 = (-x)$; $\phi_3 = x$; $\phi_4 = 4(y), 4(-y), 4(y), 4(-y)$; $\phi_5 = -\phi_1$; $\phi_6 = 2(x, y, -x, -y), 2(-y, -x, y, x)$; $\phi_7 = -\phi_6$; $\phi_8 = \text{Acq.} = (x), (-x)$.

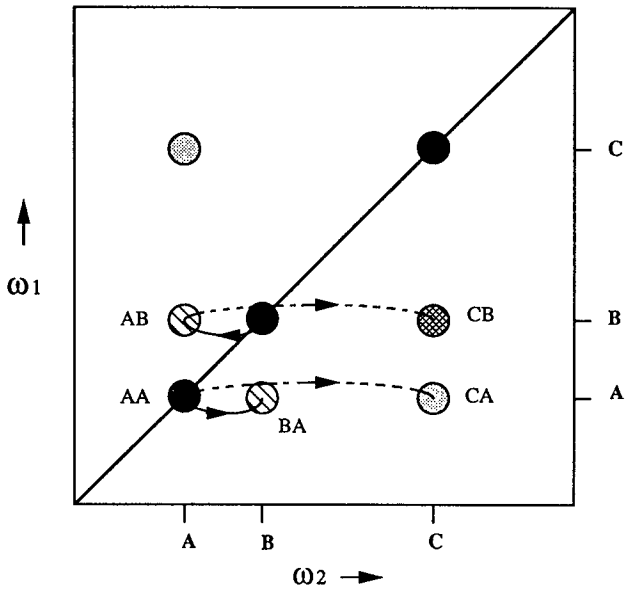

FIG. 2. Schematic representation of the asymmetry in a NOHOSS spectrum. The solid vector represents magnetization transfer during the NOE mixing period; the dashed vector represents the magnetization transfer during spin locking.

but this effect can easily be neutralized, by normalizing the cross and diagonal intensities, or by applying a theoretical correction for the excitation profile.

For quantitative analysis of exchange rates, cross-peak intensities must be corrected for chemical exchange during spin locking (ROESY-type cross peaks). The correction is obtained by a $\tau_m = 0$ experiment with the same MLEV mixing time, which reveals the relative contributions to the cross-peak intensities of the exchange during spin locking. The magnetization transfer through scalar coupling affects both the diagonal and the NOE cross peaks to the same extent. Furthermore, quantitative analysis of the data can be hampered by NOE effects that contribute to the intensities of the cross peaks between water protons and nonexchangeable protons. Direct NOE transfer is not likely. However, magnetization transfer between water and exchangeable protons followed by a NOE relay to nonexchangeable protons is possible (*25*). The amount of magnetization transferred in this way can be determined by using a NOESY sequence in conjunction with the same semiselective pulse sequence, as described for the NOHOSS experiment. These data can be used to correct NOHOSS cross-peak intensities. However, in most cases this will prove to be unnecessary. After correction of the intensities of both the diagonal and the symmetric NOE cross peaks, the exchange rates can be determined in the same way as can be done for a conventional NOESY experiment (*1–3*), preferably from a build-up series. In the case of overlapping signals the exchange rates can be determined from the asymmetric cross peaks and the symmetric HOHAHA cross peaks.

In the three-spin system ABC in Fig. 2 the spins A and C are coupled, and A and B are in exchange or are closely spaced. During the NOE mixing period magnetization

is transferred between protons A and B, affording the cross peaks AB and BA, respectively. Consequently, the magnetization present in AB and in the diagonal peak AA is transferred to proton C during the MLEV-17 mixing period, which results in the cross peaks CB and CA, respectively. Because there is in fact only one transfer from A to C, the transfer efficiencies are equal by definition, and the intensity ratio of AB to AA is equal to the intensity ratio of CB to CA. Quantitative analysis of NOESY experiments is based on comparison of the intensities of the NOE cross peak AB and the diagonal peak AA. This approach also holds for NOHOSS experiments, but similar results can be obtained by comparing the cross peak intensities of CB and CA, respectively.

As an example of the applicability of this technique a summed series of NOHOSS spectra of 50 m*M* β-methyl-cellobioside solution in $H_2O/MeOH(d_4)$ at 365K is shown in Fig. 3. The NOE mixing time was set to 0, 25, 50, 100, and 150 ms for the different

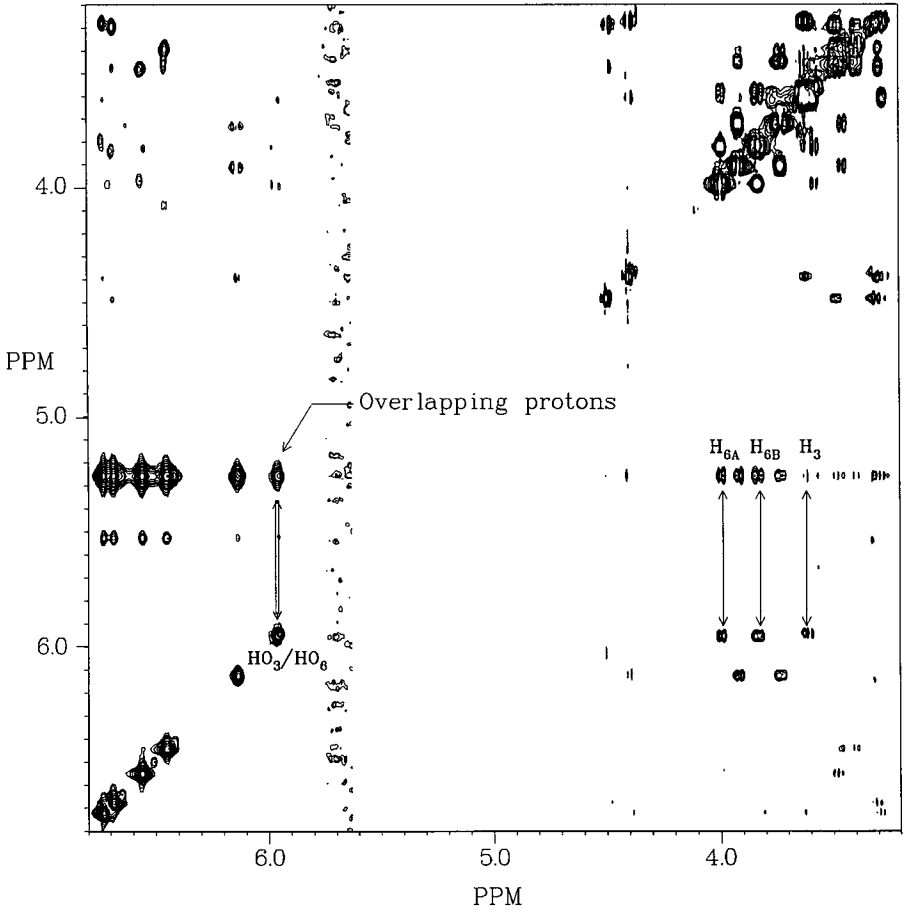

FIG. 3. Summed NOHOSS spectrum over several NOE mixing times of β-methyl-cellobioside dissolved in $H_2O/MeOH(d_4)$ 80/20 w/w. The NOE mixing time varies from 0 to 150 ms and the spin-lock pulse is 20 ms. Relevant assignments are shown in the figure.

experiments, respectively, and the spin-lock time was 20 ms in all cases. The spectral width was 2994 Hz in both dimensions. For each NOHOSS experiment 400 FIDs of 2048 data points were collected. Individual exchange rates of the two hydroxyl protons HO_3 and HO_6 resonating at about 5.9 ppm cannot be determined by a conventional NOESY experiment due to overlap. The HO_6 proton is coupled to two H_6 protons at 4.0 and 3.85 ppm, respectively, and the HO_3 proton is coupled to the H_3 proton at 3.6 ppm. Therefore, application of the NOHOSS technique allows observation of the individual exchange rates at the H_6 and H_3 resonance frequencies, as indicated in Fig. 3.

The NOHOSS method is generally applicable for measurements of exchange rates of labile protons that are in fast exchange with water protons, especially in the case of coinciding resonances of these labile protons.

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