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Normalized one-dimensional NOE measurements in isotopically labeled macromolecules using two-way cross-polarization

Elisabetta Chiarparin[a], Philippe Pelupessy[a], Brian Cutting[a], Thomas R. Eykyn[a] & Geoffrey Bodenhausen[a,b,*]
[a]*Section de Chimie, Université de Lausanne, BCH, CH-1015 Lausanne, Switzerland*
[b]*Département de Chimie, associé au CNRS, Ecole Normale Supérieure, 24 rue Lhomond, F-75231 Paris cedex 05, France*

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Abstract

A novel one-dimensional NOE experiment is presented where a selected proton is excited by two-way heteronuclear cross-polarization between protons and nitrogen-15 or carbon-13. The utility of the method is demonstrated for a sample of ^{15}N labeled human ubiquitin. Inter- and intra-residue NOEs are clearly observed in a very time-effective manner. The signal intensities can be easily normalized.

One-dimensional methods for measuring nuclear Overhauser effects (NOEs) using selective perturbations constitute an important tool for structural studies of small and medium-sized molecules (Freeman et al., 1974; Wagner and Wüthrich, 1979; Kessler et al., 1986). Recent improvements using pulsed field gradients (Stonehouse et al., 1994; Stott et al., 1995, 1997) allow one to minimize artifacts and subtraction errors. Unfortunately, none of the one-dimensional methods described so far are applicable to macromolecules with crowded proton spectra. One is usually compelled to resort to two-dimensional NOESY (Jeener et al., 1979; Kumar et al., 1981), or even to three-dimensional experiments that combine heteronuclear correlation with NOESY, so that one can benefit from the dispersion of ^{15}N or ^{13}C shifts to unravel overlapping proton signals (Cavanagh et al., 1996). In order to evaluate the cross-relaxation rates, the amplitudes of the cross peaks should be normalized by comparison with the corresponding diagonal peaks, but in practice, this is often impossible in 2D NOESY spectra because of severe overlap in the diagonal region. Heteronuclear 3D NOE techniques do not suffer from this problem, but they are very time-consuming, which makes them prone to long-term instabilities of sample and instrument, and the digital resolution is often insufficient to allow a quantitative integration of the signals.

In this communication, a novel selective one-dimensional NOE experiment is presented which is applicable to highly congested spectra. In macromolecules labeled with ^{15}N or ^{13}C, it is possible to discriminate signals that are nearly or completely degenerate in the proton domain, provided they are bound to ^{15}N or ^{13}C nuclei which differ in their resonance frequencies. This can be achieved by transferring the proton magnetization selectively to a ^{15}N or ^{13}C nucleus and back by cross-polarization, using two weak resonant RF fields with amplitudes that are smaller than the heteronuclear J couplings (Chiarparin et al., 1998). After selective excitation, the longitudinal proton magnetization is allowed to migrate through cross-relaxation to other spins in the vicinity during a mixing time τ_m. The resulting 1D spectrum corresponds to a cross-section taken through a 3D HSQC- (or HMQC-) NOESY spectrum at the ω_1 and ω_2 frequencies corresponding to the chemical shifts of a ^{15}N (or ^{13}C) nucleus and a directly bound proton. Such a 1D experiment is much less time-consuming and gives much better resolution than its 3D counter-

*To whom correspondence should be addressed.

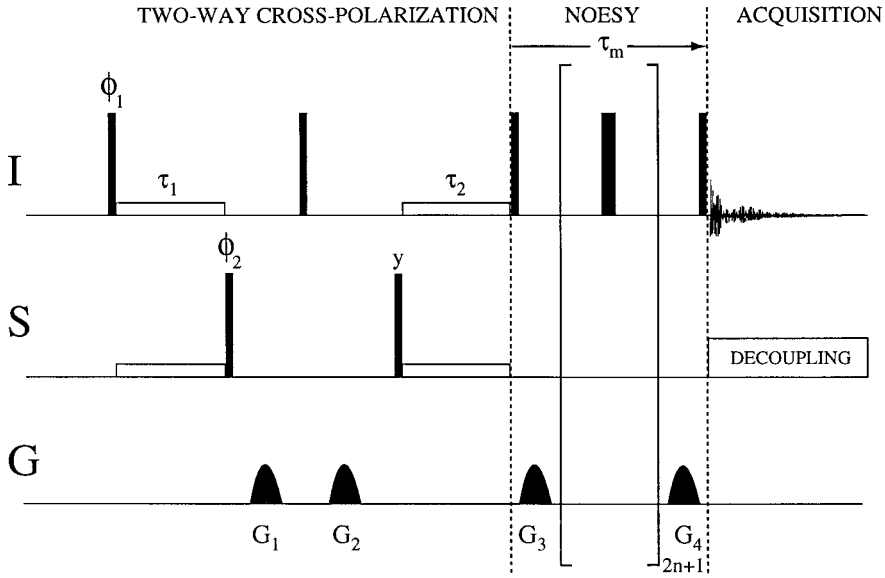

Figure 1. Pulse sequence for selective 1D NOE measurements using two-way selective cross-polarization (ISI-SCP-NOESY). For optimal cross-polarization efficiency, τ_1 and τ_2 must be set to J_{IS}^{-1} (i.e. 10.9 ms for I = ^{1}H and S = ^{15}N in amides). The carrier frequencies of the I and S channels must be set to the resonance frequencies of the selected ^{1}H and ^{15}N spins, which can be taken from an HSQC spectrum. The RF fields used for cross-polarization typically have amplitudes of approximately $J_{IS}/2$ (i.e. 45 Hz for I = ^{1}H and S = ^{15}N). A train of equally spaced π pulses is inserted during the τ_m period to suppress the recovery of longitudinal magnetization components, in particular of the solvent. The phase cycling is: Φ_1 = y, y, −y, −y, Φ_2 = y, −y, −y, y, receiver Φ_{rec} = x, −x, x, −x.

part. The method is particularly attractive when it is sufficient to quantify a limited number of NOE contacts. Indeed, a few strategically chosen NOEs are often sufficient to obtain a picture of the global structure. Complete build-up curves can be readily obtained without requiring extensive series of 3D experiments.

Figure 1 shows the pulse sequence for 1D NOESY using two-way selective cross-polarization (SCP). This may be referred to as 1D-ISI-SCP-NOESY, or, for the case in which I = ^{1}H and S = ^{15}N, 1D-HNH-SCP-NOESY. During the first cross-polarization period τ_1 (of approximate duration J_{IS}^{-1}), the magnetization is transferred from a selected source spin I (^{1}H) to a scalar-coupled partner S (^{15}N or ^{13}C). The magnetization is then stored along the *z* axis while residual transverse and longitudinal proton magnetization components (notably those of the solvent) are destroyed by spoiling gradients G_1 and G_2, applied before and after a π/2 proton pulse. During a second cross-polarization step τ_2 (again of duration J_{IS}^{-1}) the magnetization is transferred back. A π/2 pulse is used to convert I_x into a longitudinal component I_z. This conversion is essentially instantaneous, so that no cross-relaxation can occur *during* the selective inversion of the longitudinal I_z component. During the mixing time τ_m, an odd number of π pulses are applied at even intervals, sandwiched by two gradients G_3 and G_4 of opposite sign, in order to minimize the recovery of longitudinal components that do *not* stem from the source of interest (Stott et al., 1995). The resulting longitudinal magnetization can be converted into observable transverse magnetization by a simple π/2 pulse. There is no need for appending further water suppression sequences such as WATERGATE (Sklenar et al., 1993) or excitation sculpting (Hwang and Shaka, 1995), which would lead to an attenuation of solute signals (e.g. H^α protons in proteins) in the vicinity of the solvent resonance. The destruction of transverse and longitudinal solvent magnetization components between the two cross-polarization steps, combined with the series of π pulses in the mixing time, allows one to achieve excellent water suppression. The sensitivity of the experiments is primarily determined by $T_{1\rho}$ relaxation during the two cross-polarization steps, which have an overall duration $\tau_1 + \tau_2 = 2/J_{IS}$. In ubiquitin, we have found that 60% of the magnetization was retained during two-way cross-polarization (Pelupessy et al., 1998). The relaxation losses are comparable to those in two-way refocussed INEPT, where the decay of the signal is given by $\exp(-t/T_2^I)\exp(-t/T_2^S)$, with $t = 1/J_{IS}$ (Majumdar and Zuiderweg, 1994).

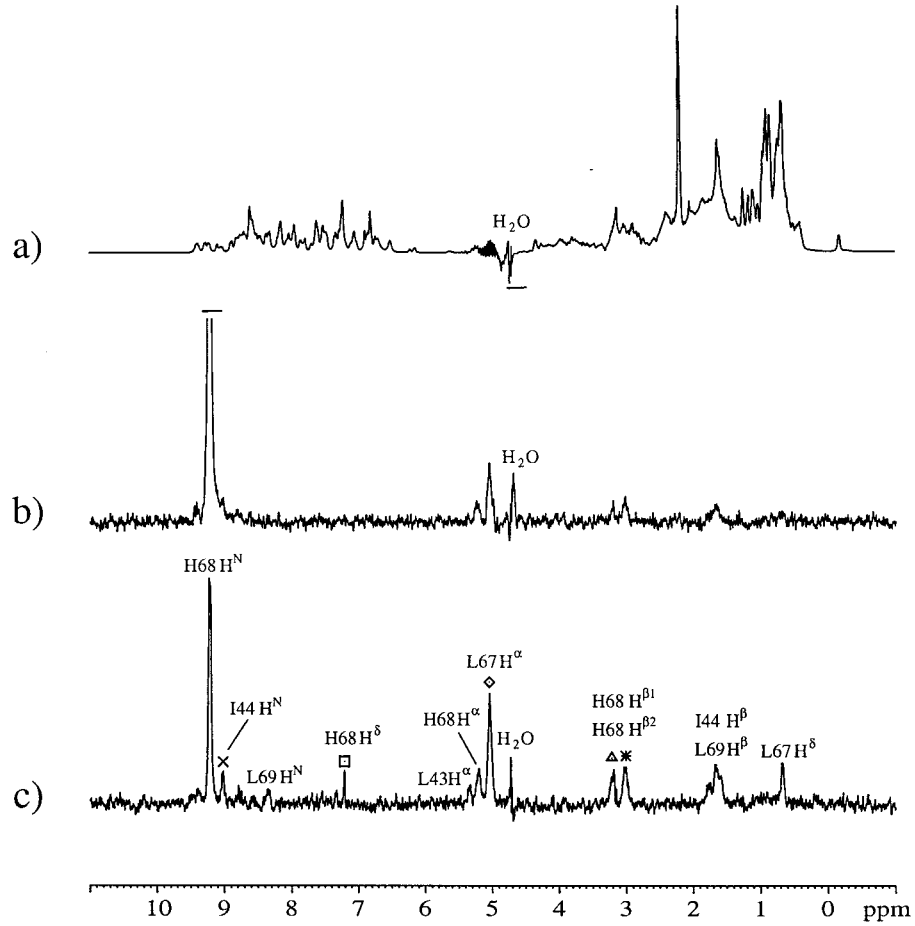

Figure 2. (a) Conventional 1D spectrum of ^{15}N labeled human ubiquitin at 303 K and 400 MHz with ^{15}N decoupling. The solvent signal was suppressed by excitation sculpting. (b) and (c) 1D-HNH-SCP-NOESY spectra obtained by excitation of the H^N amide proton of H68 in ubiquitin followed by mixing times $\tau_m = 64$ ms and 400 ms, respectively. The signal of the 'source' spin corresponds to a diagonal peak in a 2D NOESY spectrum or to a 'parent' peak in a 3D HSQC-NOESY spectrum. All other peaks correspond to protons in the vicinity of the source spin. An exponential window function with a decay constant of 3 Hz has been applied to all spectra, without using any further data manipulation such as deconvolution.

All experiments have been carried out with a Bruker 400 MHz DRX spectrometer at 303 K with a 1.5 mM sample of ^{15}N-labeled human ubiquitin (VLI Research) in $H_2O : D_2O = 9 : 1$ buffered at pH = 4.5 with 20 mM perdeuterated acetic acid. Figure 2a shows a ^{1}H spectrum of ^{15}N labeled ubiquitin obtained with excitation sculpting (Hwang and Shaka, 1995) and ^{15}N decoupling. Figures 2b and 2c show the results of selective two-way cross-polarization (from amide H^N to ^{15}N and back to H^N) in histidine 68 (H68), followed by laboratory-frame NOE with mixing times $\tau_m = 64$ ms and 400 ms, respectively. The resulting 1D NOE spectra show the 'source' H^N of H68 and various 'target' spins in its vicinity. Even signals close to the water peak can readily be quantified. The peaks have been assigned according to Wang et al. (1995) and DiStefano and Wand (1987). One can recognize not only signals belonging to H68 and its neighbours L69 and L67 (β sheet number 5), but also signals originating from non-contiguous amino acids I44 and L43 (β sheet number 3). These NOEs yield information on the relative positions of the two β sheets (Cavanagh et al., 1996).

Figure 3a shows a detail of the X-ray structure of ubiquitin in the vicinity of the amide proton of H68. The X-ray data (Vijay-Kumar et al., 1987) were treated with the program Sybyl (Rohm and Haas company) by minimizing the energy of the structure using a Kollman United Force Field (Weiner et al., 1984) after insertion of the protons. A total of 11 build-

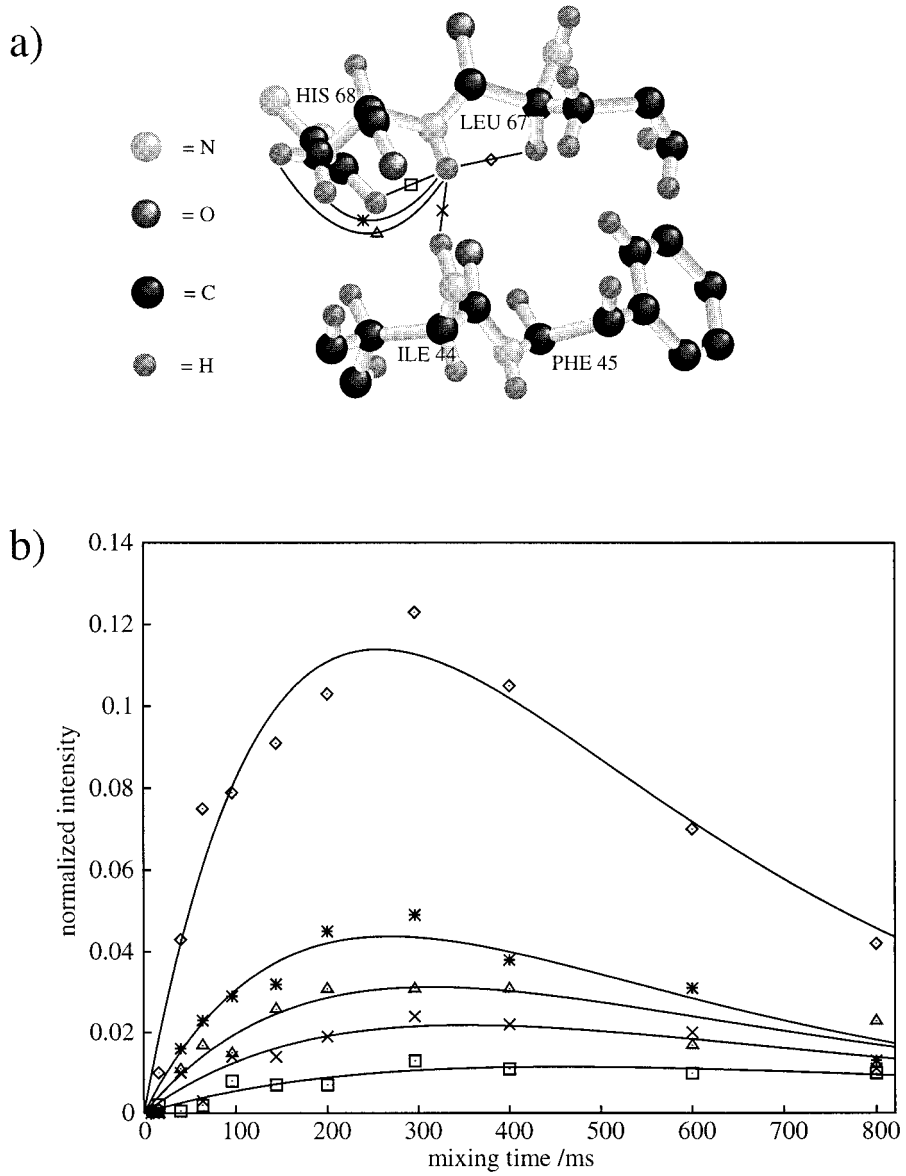

Figure 3. (a) Detail taken from the X-ray structure of human ubiquitin showing the neighbourhood of the H^N 'source' proton of H68, with some competing cross-relaxation pathways. (b) NOE build-up curves corresponding to these cross-relaxation pathways, normalized with respect to the intensity of the 'source' peak extrapolated back to $\tau_m = 0$. The shortest recorded mixing time τ_m was 8 ms. The intensities were determined by 1D integration of the peak areas. The symbols are consistent with those used in Figure 2c.

up curves have been measured. For clarity, only five curves are shown in Figure 3b. The cross-peak intensities can be readily normalized by division through the amplitude of the 'source peak' of the amide proton of H68 extrapolated back to $\tau_m = 0$. This extrapolation is straightforward, since the minimum experimental duration of the mixing period was $\tau_m = 8$ ms (8 intervals of 1 ms each, separated by 7 π pulses) and since there can be no artifacts due to zero-quantum coherences associated with homonuclear scalar couplings between the protons. Thus normalization, one of the least satisfactory aspects of the interpretation of 2D NOESY spectra (Neuhaus and Williamson, 1989), becomes a trivial matter.

Experiments such as shown in Figures 2b and 2c require typically 1 h each for a 1.5 mM solu-

tion of ubiquitin at 400 MHz. The complete series in Figure 3b required 10 h. Such curves allow one to observe the build-up behaviour without the need for recording 3D NOESY experiments with different mixing times. By comparison, 10 different 3D HSQC-NOESY spectra require about 20 days under these conditions.

1D-ISI-SCP-NOESY makes it possible to supplement time-consuming 3D NOESY experiments by recording series of quick 1D experiments. The method provides accurate signal intensities and does not require any solvent suppression techniques that may distort amplitudes of signals in the vicinity of the solvent resonance.

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