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Excitation of Selected Proton Signals in NMR of Isotopically Labeled Macromolecules

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In isotopically labeled macromolecules, it is possible to excite the signal of a selected proton by shuttling magnetization back and forth between the chosen proton and a heteronucleus such as ^{13}C or ^{15}N, using two-way doubly selective heteronuclear cross-polarization. Selective excitation of a chosen proton can be followed by homonuclear coherence transfer to identify side-chain resonances of the corresponding amino acid in proteins. The resulting one-dimensional experiments yield information that can usually only be obtained from three-dimensional HSQC–TOCSY spectra. The method also provides efficient suppression of solvent signals without affecting resonances close to the solvent peak. © 1999 Academic Press

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Much has been written about the virtues of selective excitation in magnetic resonance (*1*). Crowded spectra can be unraveled in exquisite detail (*2–4*). Band-selective versions of multidimensional spectra can provide excellent resolution (*5, 6*). Cumbersome three- or four-dimensional spectra can be reduced to two- or one-dimensional spectra (*1, 7*). Doubly selective homonuclear coherence transfer allows one to measure relaxation rates of individual protons (*8, 9*). Synchronous nutation (*10*) and doubly selective inversion (*11*) make it possible to measure Overhauser effects while suppressing spin diffusion (*12*). All of these selective methods normally rely on a variety of amplitude- and phase-modulated pulses (*13, 14*).

Unfortunately, these ideas are not truly suitable for macromolecules with severely overlapping proton spectra. The more crowded the spectra, the longer the selective pulses need to be and the greater the toll taken by transverse relaxation and evolution under homonuclear couplings during the pulses. Typically, a selective Gaussian 270° pulse must be as long as 30 ms to excite a multiplet of 30 Hz width without phase distortion, leading to prohibitive losses in signal amplitude in macromolecules. For this reason, one is usually compelled to resort to cumbersome three- and four- dimensional methods (*15*).

In this Communication, we propose a way to extend the benefits of selective experiments to isotopically labeled macromolecules. Selective heteronuclear cross-polarization using two weak radio-frequency fields has been shown to be very effective in transfering magnetization from a selected proton to a scalar-coupled heteronucleus (*16*). Two-way coherence transfer $I \rightarrow S \rightarrow I$ can be carried out within an interval on the order of $2\tau = 2(^1J_{IS})^{-1}$ (e.g., $2\tau = 20$ ms if $^1J_{IS} = 100$ Hz). Selective spin-locking effectively decouples homonuclear *J*-couplings, which helps to minimize signal losses.

The principle is illustrated in Fig. 1. The I_x magnetization of a nucleus that one wishes to excite selectively (usually ^1H) is transformed by cross-polarization into S_x magnetization of an *S* nucleus (^{13}C or ^{15}N). Provided that the RF fields applied to both I and S spins are weak (typically about $^1J_{IS}/2$), the Hartmann–Hahn condition (*17*) need not be fulfilled accurately and inhomogeneous RF fields do not significantly affect the transfer efficiency (*16*). Both intervals $\tau_1 = \tau_2$ are set approximately to $(^1J_{IS})^{-1}$. The carrier frequencies of the I and S channels must be set to the chemical shifts of selected I and S resonances, which can be taken from a heteronuclear correlation HSQC spectrum such as that shown in Fig. 2 (in this case, ubiquitin, with 76 amino acids, 8.5 kDa, $\tau_c \sim 4$ ns at 300 K, and a typical amide proton $T_2 \sim 40$ ms). In analogy to techniques employing broadband cross-polarization (*18*), gradients and RF pulses are used to destroy residual transverse and longitudinal proton magnetization (in particular solvent magnetization), while the ^{15}N magnetization of interest is stored along the *z* axis. Note that the suppression of the solvent signal is excellent without resorting to selective proton pulses and gradient labeling methods.

Figure 3c shows the amide proton of glutamine Q62 in ubiquitin detected immediately after the second cross-polarization step (i.e., by skipping the TOCSY sequence in Fig. 1). The success of the method depends on the selectivity of the RF fields during two-way doubly selective cross-polarization, and on the fact that the relevant cross peak must be resolved in the HSQC spectrum (a condition which must also be fulfilled for the success of 3D experiments). In Fig. 2, the Q62 signal appears fairly close to the D21 signal (shifted by 0.3 and 0.8 ppm in the ^1H and ^{15}N dimensions, which at 300 MHz amounts to 100 and 25 Hz, respectively) (*19, 20*). In Fig. 2, a simulated

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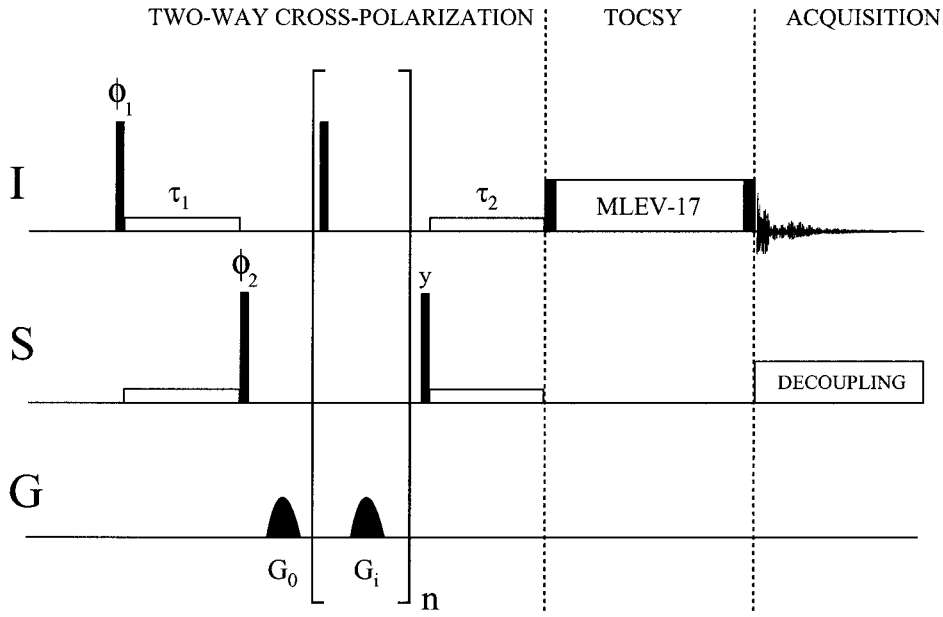

FIG. 1. Sequence for excitation of a selected proton in an isotopically labeled macromolecule, followed by total correlation spectroscopy (TOCSY). Narrow vertical bars indicate 90° pulses. The duration of the two cross-polarization intervals $\tau_1 = \tau_2$ is set in the vicinity of $({}^1J_{IS})^{-1}$ (i.e., 10.9 ms for I = ^{1}H and S = ^{15}N in amides with $J_{IS} = 92$ Hz). The carrier frequencies of the I and S channels are set to the chemical shifts of selected I and S resonances. The two weak RF fields have approximately equal amplitudes on the order of ${}^1J_{IS}/2$ (ca. 40 Hz). Unless indicated otherwise, the phases of the pulses are along the *x* axis. The following four-step phase cycle is used: $\phi_1 = y, y, -y, -y$, $\phi_2 = y, -y, y, -y$, receiver = $x, -x, -x, x$. The gradient G_0 allows one to destroy residual transverse proton magnetization, while the combination of *n* 90° pulses on the proton channel followed by *n* gradients G_i allows one to destroy longitudinal proton magnetization (typically, $n = 2$ in our experiments). The ^{15}N magnetization of interest is stored along the *z* axis during this purging procedure. If the signal is detected immediately after the second cross-polarization step (i.e., if the TOCSY sequence is skipped), one obtains the signal of only the selected proton. If a TOCSY sequence is inserted prior to signal acquisition, one obtains signals of all protons belonging to the same scalar coupling network. The MLEV-17 pulse sequence is preceded and followed by trim pulses.

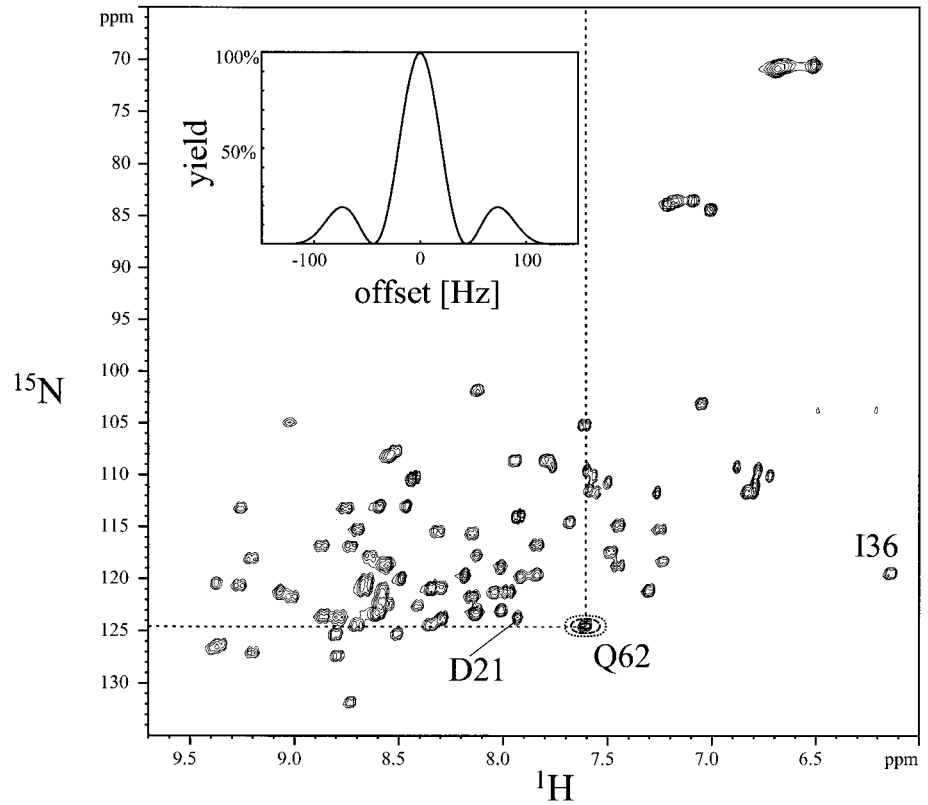

FIG. 2. Experimental HSQC spectrum of 1.5 mM ^{15}N-labeled ubiquitin (VLI Research) in H_2O:D_2O = 9:1 obtained at 300 MHz. A calculated two-dimensional offset dependence of the efficiency of heteronuclear two-way cross-polarization is overlaid with the Q62 cross peak. The contour lines correspond to 20, 50, and 90% yield of excitation. In the top left corner a one-dimensional cross section at the nitrogen resonance frequency is shown. The heteronuclear scalar coupling used in the simulations was 90 Hz, while the spin-locking fields had amplitudes of 40 Hz each and a duration of 10.9 ms. The efficiency of excitation drops below 50% at offsets of ±22 Hz and below 20% at offsets of ±30 Hz in either proton or nitrogen-15 dimensions.

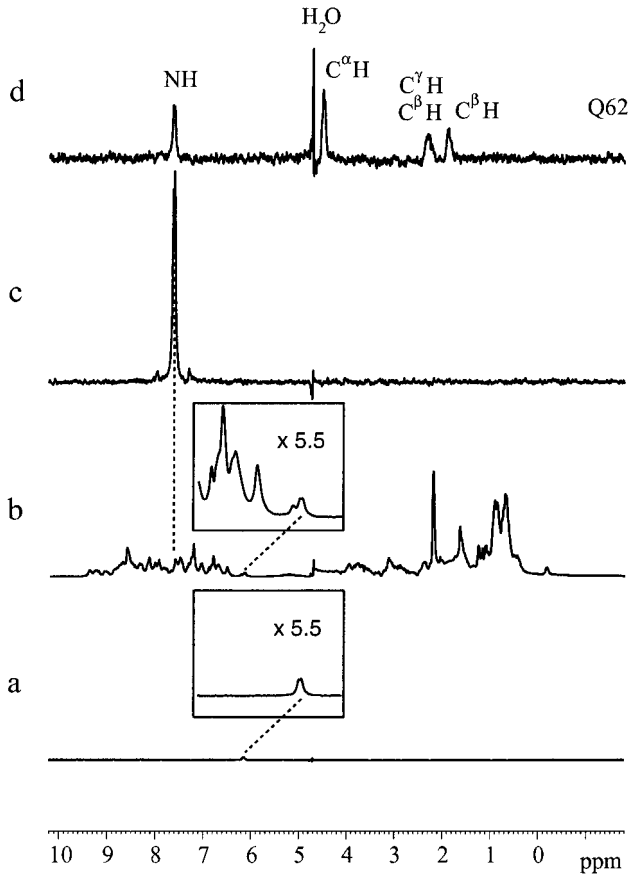

FIG. 3. (a) Selective excitation of the amide proton of isoleucine I36 in ubiquitin using the sequence of Fig. 1 without TOCSY and with two solvent suppression cycles ($n = 2$ in Fig. 1). The RF amplitudes during cross-polarization were about 40 Hz ($^1J_{IS} = 92$ Hz) for both ^{1}H and ^{15}N channels. (b) Conventional proton spectrum of ubiquitin at 300 MHz recorded with ^{15}N decoupling and excitation sculpting using two selective 180° pulses of 6 ms each and four gradients of 1 ms each (overall duration 29 ms). The intensities of the I36 amide proton signals in (a) and (b) are equal, as shown by the enlargements. (c) Selective excitation of the amide proton of glutamine Q62 using the same RF amplitudes as in (a). (d) Signals obtained with TOCSY (mixing time 65 ms, two trim pulses of 2 ms each), showing all protons belonging to the coupling network of Q62. The RF amplitude during the MLEV-17 sequence was 2.5 kHz. For spectra (c) and (d), 512 scans were accumulated in 15 min. The vertical scale of (d) is twice that of (c). Spectra (a) and (b) were obtained with 64 scans in 2 min and identical receiver gain.

two-dimensional offset profile (calculated with the GAMMA program (*21*) using parameters corresponding to experimental conditions, without considering relaxation effects) is superimposed on the Q62 peak. A one-dimensional cross section at the nitrogen resonance is shown in the top left corner. At offsets of ± 22 Hz in either proton or nitrogen dimensions, the efficiency of the two-way transfer drops to less than 50%. Even at 300 MHz, the selectivity of our experiments is sufficient. If the signals were contaminated by nearby cross peaks, the selectivity could be improved by optimizing the shapes of the RF fields during cross-polarization or by using higher static magnetic fields.

In Figs. 3a and 3b, the amplitude of the amide proton of isoleucine I36 in ubiquitin obtained with two-way cross-polarization (which automatically achieves solvent suppression) has been compared with a signal recorded after simple 90° proton excitation, combined with excitation sculpting (*22*) to suppress the solvent peak. The two amplitudes are similar, indicating that signal losses incurred during two-way cross-polarization (22 ms total duration) are comparable to losses suffered during excitation sculpting (sequence of 29 ms). The absolute loss has been measured by applying our selective excitation scheme twice in a row and dividing the amplitude of the resulting signal with that of the regular scheme (results not shown). The losses were 40% in ubiquitin and are almost entirely due to relaxation (*16*).

Selective excitation can be followed by a wide variety of techniques for coherent or incoherent transfer of magnetization. In particular, one can insert a mixing period where longitudinal or transverse magnetization is allowed to migrate through cross-relaxation in the manner of NOESY or ROESY. For the sake of illustration, we have chosen to insert a sequence for total correlation spectroscopy (TOCSY) prior to signal acquisition, so that one obtains signals for all protons belonging to the same scalar coupling network of the proton that has been excited selectively (*2–4*). In Fig. 3d, an MLEV-17 sequence of 65 ms duration was preceded and followed by trim pulses of 2 ms duration each. No further measures were needed to suppress the water resonance. One can identify signals of the NH, $C^{\alpha}H$, two $C^{\beta}H$'s, and two $C^{\gamma}H$'s of glutamine Q62 in ubiquitin (*19, 20*). The $C^{\alpha}H$ signal is very close to the water signal. The total integrated intensity of these signals amounts to 56% of the intensity of the Q62 NH signal in Fig. 3c, thus allowing one to measure losses due to relaxation in the course of the homonuclear Hartmann–Hahn (TOCSY) transfer. If the ^{15}N nuclei were not decoupled during proton acquisition, one can determine short- and long-range couplings $^nJ(^{15}\text{N}, ^1\text{H})$ from the multiplets. The information in Fig. 3c is similar to what could be obtained from 3D HSQC–TOCSY spectra, but only required 15 min at 300 MHz. Quantitation of cross-relaxation (NOEs and ROEs) is equally straightforward (*23*).

Our approach to selective excitation exploits the presence of isotopic labels in combination with two-way doubly selective heteronuclear cross-polarization. The method provides excellent sensitivity and efficient solvent suppression. For small- and medium-size molecules such as cyclosporin, the method is also applicable without isotope labeling.

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