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Enhanced sensitivity of rapidly exchanging amide protons by improved phase cycling and the constructive use of radiation damping

Wolfgang Jahnke and Horst Kessler*

Institut für Organische Chemie und Biochemie, TU München, Lichtenbergstraße 4, D-85747 Garching, Germany

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SUMMARY

Two alternative, general methods are presented that lead to enhanced signal intensity of rapidly exchanging protons. Both methods work by avoiding saturation of the water resonance, and are convenient to implement since they do not use any selective pulses. One method carefully chooses proton pulse phases and gradient strength and position in such a way that the water is realigned along the +z axis at the beginning of the acquisition time. An alternative method is proposed for cases where the pulse sequence does not allow such phase cycling. The latter uses radiation damping to bring water back to the +z axis 20–30 ms after acquisition. The methods are applied to the triple-resonance experiments HNCA, HNCO and HN(CO)CA. Both methods require pulsed B_0 field gradients and can result in higher signal intensity by a factor of two or more.

The sensitivity of NMR experiments is a major concern in the design of all modern pulse sequences that are applied to the study of biopolymers. Important recent developments that increase the sensitivity of NMR experiments include the sensitivity enhancement concept proposed by Rance and co-workers (Cavanagh and Rance, 1990; Palmer et al., 1991; Kay et al., 1992; Schleucher et al., 1994), and the concept of nonlinear sampling (Schmieder et al., 1993). These techniques increase the signal-to-noise ratio for all peaks in the spectrum. In practice, it is often found when assigning spectra of a biopolymer that most resonances are sufficiently strong to yield correlations in various experiments. However, there is usually also the small percentage of weak signals where important cross peaks are missing in several experiments. Some of these weak resonances may belong to amide protons not involved in regular secondary structure that exchange rapidly with the solvent, and it is often this small fraction of all signals that hamper the

*To whom correspondence should be addressed.

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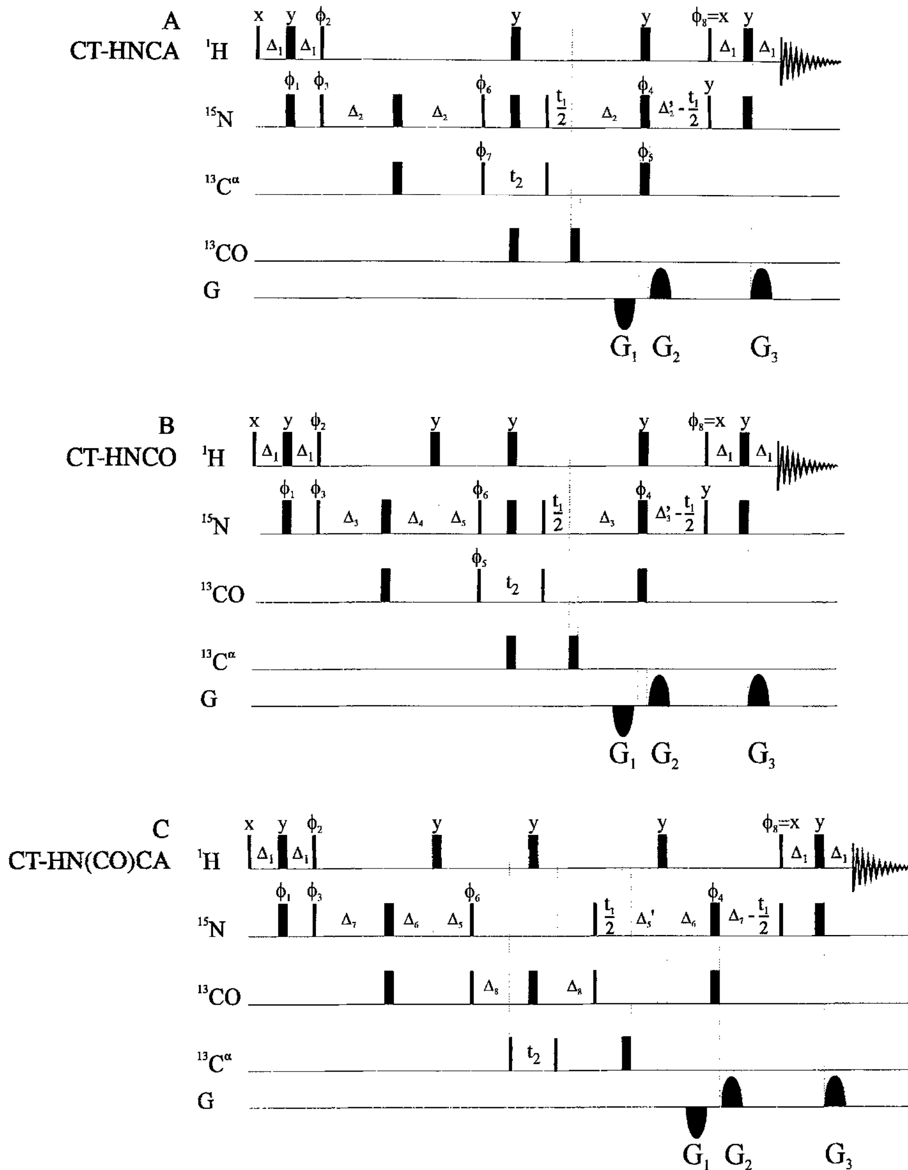

Fig. 1. Pulse sequences for modified triple-resonance experiments with enhanced sensitivity for rapidly exchanging amide protons: CT-HNCA (A), CT-HNCO (B) and CT-HN(CO)CA (C). The enhanced sensitivity is achieved by not saturating water before acquisition. Thin bars represent 90° pulses, thick bars represent 180° pulses. Pulses where no phase is given are applied along the x axis. Delays are as follows: $\Delta_1 = 2.25$ ms, $\Delta_2 = 11$ ms, $\Delta'_2 = \Delta_2 + \tau_{180°}(^{13}C)$, $\Delta_3 = 13.75$ ms, $\Delta'_3 = \Delta_3 + \tau_{180°}(^{13}C)$, $\Delta_4 = 11$ ms, $\Delta_5 = 2.75$ ms, $\Delta'_5 = 2.75$ ms $- \tau_{180°}(^{13}C)$, $\Delta_6 = 7.25$ ms, $\Delta_7 = 10$ ms $+ \tau_{180°}(^{1}H)$. Note that for good results, the delays have to be set as described. Pulsed field gradients are sine-bell shaped and have a duration of 1.5 ms, which includes 0.5 ms recovery delay for the B_0 field. Gradient strengths are $G_1 = -39.6$, $G_2 = 39.4$, $G_3 = 8$, where a gradient strength of 100 corresponds to ca. 50 G/cm at the center of the gradient. Gradients G_1 and G_2 need exact calibration. Phase cycling is as follows: For (A): $\phi_1 = x,-x$, $\phi_2 = y,-y$, $\phi_3 = x$, $\phi_4 = 4(x),4(y),4(-x),4(-y)$, $\phi_5 = 16(x),16(-x)$, $\phi_6 = 16(y),16(-y)$, $\phi_7 = 2(x),2(-x)$, receiver $= 2(x,-x,-x,x,-x,x,x,-x),2(-x,x,x,-x,x,-x,-x,x)$. For (B) and (C): $\phi_1 = x,-x$, $\phi_2 = y,-y$, $\phi_3 = x$, $\phi_4 = 4(x),4(y),4(-x),4(-y)$, $\phi_5 = 2(x),2(-x)$, $\phi_6 = x,-x$, receiver $= 2(x),4(-x),2(x)$.

assignment and take the majority of the spectroscopist's time, or constitute the bottleneck during an automated assignment procedure.

We have recently proposed a method, MEXICO, to identify these rapidly exchanging amide protons and measure their exchange rates (Gemmecker et al., 1993). Here we describe an approach that increases signal strength predominantly for these weak signals, where improved sensitivity is of outstanding importance.

Protons may exchange between protein and water on a time scale comparable to the duration of a single scan in an NMR experiment. The signals of these rapidly exchanging protein protons are attenuated or broadened, due to magnetization transfer to water during the pulse sequence, including acquisition. However, an equally or more important contribution to the attenuation of these signals is the fact that, if the water has been saturated prior to acquisition, protons that exchange from water to the protein during the relaxation delay bring with them only a fraction of the equilibrium longitudinal magnetization necessary for the next scan. This is due to the long T_1 relaxation time of water protons (4–5 s), which is much longer than the T_1 of protein protons and typically much longer than the relaxation delay between successive scans. The importance of not saturating water in protein NMR has recently been pointed out by Grzesiek and Bax (1993) and Stonehouse et al. (1994), and it was found that the signal-to-noise ratio for weak signals could be increased when water was not saturated. While Grzesiek and Bax used selective pulses to manipulate the water magnetization separately from the protein protons, Stonehouse et al. achieved the same effect in a standard HSQC by proper choice of pulse phases. In this communication, we extend the latter method and apply it to triple-resonance experiments, demonstrating that a considerable increase in signal-to-noise for rapidly exchanging amide protons can be achieved by these simple modifications.

Figure 1 shows the modified pulse sequences of the constant-time experiments HNCA, HNCO and HN(CO)CA (Grzesiek and Bax, 1992). In all pulse sequences, water saturation is minimized by a proper choice of proton pulse phases: The first 90° proton pulse with phase x aligns the water magnetization along the −y axis, where it remains as the carrier is set to the water frequency. Water magnetization is hardly affected by the subsequent proton pulses with phase ± y. It is then inverted by the 90° proton pulse with phase ϕ_8 = x, and subsequently returned to the +z axis by the last proton 180° pulse. Any remaining transverse component is defocussed by the pulsed field gradient G_3. Thus, excellent water suppression is achieved, together with minimum water saturation.

The pulse sequences of Fig. 1 were used to record triple-resonance spectra of a 2 mM sample of the IIA^{Man} domain of the mannose transporter of *Escherichia coli* (Seip et al., 1994), a 31 kDa homodimer that was shown to possess a number of rapidly exchanging amide protons (Gemmecker et al., 1993). Figure 2 compares the first 2D planes of an HNCA experiment, once recorded without saturation of water (panel A, pulse sequence of Fig. 1A), and once recorded with saturation of water (panel B, pulse sequence of Fig. 1A, but phase ϕ_8 = y). The displayed section contains two peaks that correspond to very rapidly exchanging amide protons, as revealed by the MEXICO spectrum (not shown). The fact that these two rapidly exchanging amide protons give rise to cross peaks in the HNCA spectrum without water saturation (Fig. 2A), but not in an HNCA spectrum recorded in the conventional way (Fig. 2B) strongly emphasizes the advantage of not saturating water in triple-resonance experiments.

It was found that proper refocussing of the water magnetization depends critically on the exact

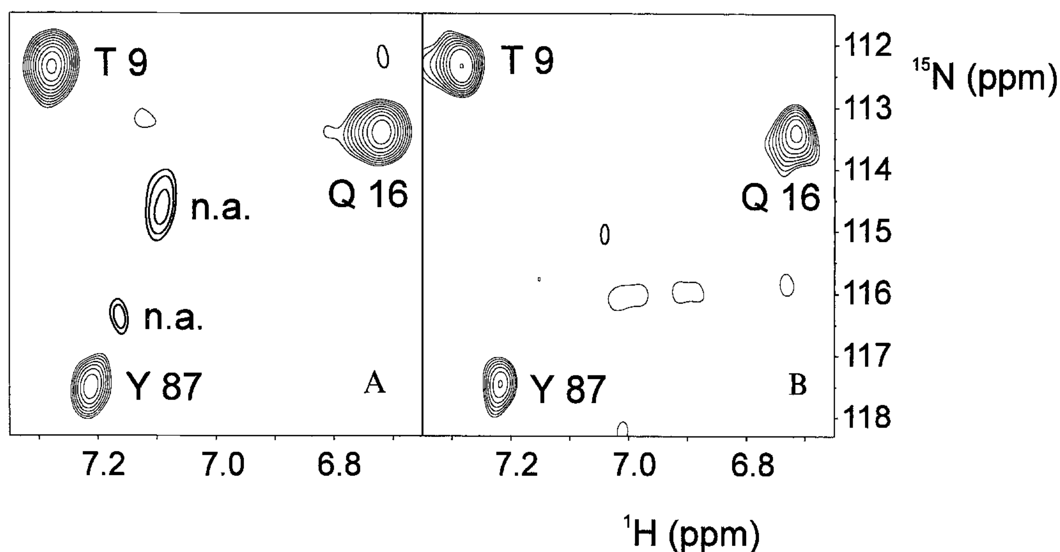

Fig. 2. Regions of the first 2D planes of HNCA spectra, recorded (A) without saturation of water, according to the pulse sequence of Fig. 1A, and (B) with saturation of water, according to the pulse sequence of Fig. 1A, but with phase ϕ_8 set to y. Both spectra were otherwise recorded, processed, and plotted identically. Negative levels are indicated by bold lines. Assignments are indicated in the spectra, and 'n.a.' refers to peaks that have not yet been assigned. Note that these peaks, which involve rapidly exchanging amide protons, are only visible in the HNCA spectrum without water saturation, even though both spectra are plotted at the noise level. Both spectra were acquired on a Bruker AMX600 spectrometer at 600 MHz proton frequency, with triple-resonance equipment and pulsed field gradients along the z axis. The sample was a 2 mM solution of the mannose permease domain IIA^{Man}, a homodimer of 31 kDa, at pH 7.5 and 310 K (Seip et al., 1994).

tuning of the gradient strengths. On our spectrometer, the theoretical gradient values of $G_1 = -39.6$ and $G_2 = 39.6$ (with 100 being ca. 50 G/cm at the center of the gradient) refocussed water only about 50%, while with a setting of G_2 to 39.4, almost the entire water magnetization was refocussed. An exact tuning of gradient strengths is thus essential for the success of the experiment.

The method of realigning water along the +z axis by a proper choice of proton pulse phases can be applied to a variety of multidimensional heteronuclear NMR experiments. There are, however, a few cases where application of these principles is not possible, but water saturation has to be avoided for accurate results. One of these cases is the measurement of heteronuclear ^{15}N-{^{1}H} NOE values (Kay et al., 1989; Clore et al., 1990; Stone et al., 1992), where only one proton 90° pulse is applied and the water can thus not be realigned along the +z axis by proper phase cycling. The second method, proposed below, helps to alleviate this problem, although it does not work as well as the one described above. For comparison, it is explained with the HNCA pulse sequence of Fig. 1A, where phase ϕ_8 is now y, so that water is *not* realigned along the +z axis before acquisition.

Water protons, with their high concentration as a solvent, show a phenomenon known as radiation damping (Abragam, 1961; Freeman, 1987). Due to coupling with the receiver coil, any transverse component of the water magnetization induces a voltage in the receiver coil that acts just as a pulse to bring the water magnetization back to the +z axis. This feature is often an annoyance, since it can result in an unpredictable state of the water magnetization that leads to

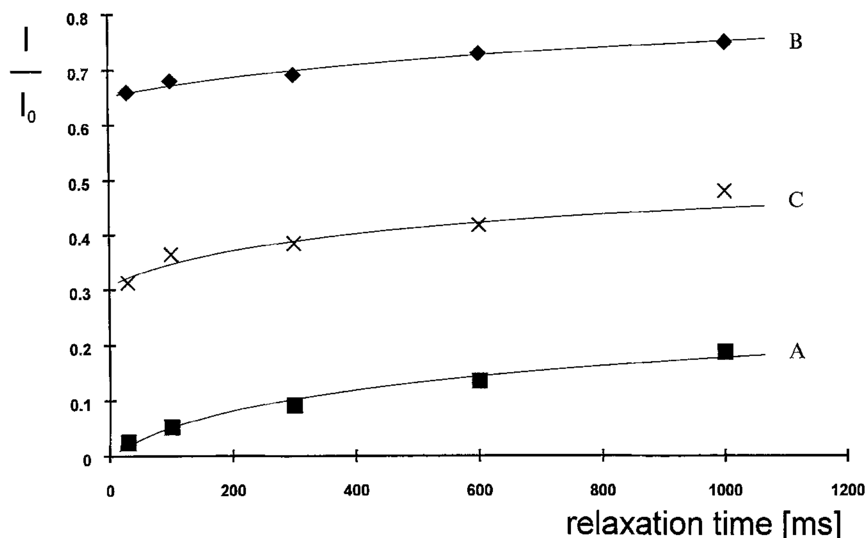

Fig. 3. Sampling of longitudinal water magnetization at different times during the relaxation delay in an HNCA experiment. (A) After saturation of the water resonance by pulsed field gradients prior to acquisition, as in a conventional HNCA experiment (pulse sequence as in Fig. 1A, but phase ϕ_8 = y). (B) Without water saturation, achieved by proper phase cycling as proposed in the text, with the pulse sequence of Fig. 1A. (C) Using radiation damping to bring water back to the +z axis after acquisition, after a single scan of the pulse sequence of Fig. 1A, but with phase ϕ_8 = y and a refocussing gradient $G_4 = -G_3$ after acquisition.

spectral artifacts. Here, however, it can be usefully applied for the enhancement of signals from rapidly exchanging amide protons. Radiation damping occurs only when all water magnetization is in-phase, and is suppressed after a pulsed field gradient since the dephased water magnetization does not yield a net current to induce a voltage in the receiver coil. Thus, after the first three gradients in the pulse sequence of Fig. 1A (where phase ϕ_8 is now y), the water magnetization is transverse, but does not radiation damp since it is dephased. However, if a refocussing gradient $G_4 = -G_3$ is applied right after acquisition, all water magnetization is in-phase again, and due to radiation damping, it goes back to the +z axis within 20 or 30 ms. The desired nonsaturated state of water is thus achieved, independent of the number and phases of proton 90° pulses in the pulse sequence.

Unfortunately, due to diffusion during the acquisition time, the water magnetization cannot be entirely refocussed and thus does not have its full equilibrium value after radiation damping has occurred. As expected, the refocussing is more complete for short acquisition times and for lower temperatures. We have sampled the longitudinal water magnetization at different times during the relaxation delay, and the result is shown in Fig. 3. Curve A represents the longitudinal water magnetization after one scan of a conventional HNCA experiment with water suppression and saturation by pulsed field gradients. There is almost no longitudinal water magnetization left after acquisition, and it grows only slowly due to the water T_1 of about 4–5 s. After 1 s, when typically the next scan begins, water has reached only about 18% of its equilibrium longitudinal magnetization. If the water is realigned along the +z axis, as proposed in the pulse sequence of Fig. 1A, it already reaches about 68% of its equilibrium value right after acquisition, as shown in curve B. This value increases to about 75% by T_1 relaxation before the next scan. Chemical

exchange with amide protons thus brings almost the full magnetization to the amide proton and does not a priori attenuate the amide signal. Curve C was sampled using the pulse sequence of Fig. 1A, but with phase ϕ_8 being y, so that water is not realigned along the +z axis by phase cycling before acquisition. Instead, a refocussing gradient was applied after the acquisition time (which was 50 ms, at 310 K). As can be seen, about 35% of the water magnetization is back at the +z axis after about 30 ms, due to radiation damping.

During the relaxation delay, another 10–15% relax by T_1 relaxation, so that almost 50% of the equilibrium longitudinal water magnetization is present before the next scan. Unfortunately, in the steady state of subsequent pulses, this number reduces to about 30%, but that still means an improvement compared to the 18% observed in the conventional HSQC.

We have proposed two methods that produce spectra with clearly enhanced sensitivity for rapidly exchanging protons, i.e., for those protons where increased sensitivity is most important. Both methods do not employ selective pulses and are straightforward to implement. The resulting sensitivity enhancement is thus virtually 'for free', and can be applied to a variety of heteronuclear multidimensional NMR experiments. These modifications may lead to more complete assignments of protein spectra. If ^{15}N-edited NOESY spectra are recorded in a similar manner, structure determination may become more accurate, since more NOEs involving rapidly exchanging amide protons will be detected to define the protein structure in regions that would otherwise be considered ill-defined or flexible.

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