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Article

SWET for secure water suppression on probes with high quality factor

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Abstract

Water suppression by selective preirradiation is increasingly difficult to achieve on probeheads with high quality factor because of the opposing forces of radiation damping. Here we show that a simple modification to the WET scheme provides reliable water suppression in aqueous solutions of proteins and peptides with minimal saturation of the H^{α} protons. The scheme is shown to work also with dilute peptide solutions. It is recommended to maintain the water suppression during the evolution time of COSY experiments by weak selective irradiation that causes only minimal Bloch-Siegert shifts. The new water-suppression scheme suppresses the water magnetization by spatial scrambling. Traditional water suppression by preirradiation is similarly based more on water scrambling due to the radiofrequency inhomogeneity than on relaxation effects.

Abbreviations: DANTE – delays alternating with nutations for tailored excitation; rf – radiofrequency; SWET – secure WET; WANTED – Water selective DANTE using gradient; WET – water suppression enhanced through T_1 effects

Introduction

Among many different water suppression schemes (Price, 1999), water suppression by selective irradiation (Campbell et al., 1974) stands out as one of the most universally applicable schemes. The high quality factor, Q, of modern probeheads on high-field NMR spectrometers, however, makes water suppression by selective irradiation an increasingly difficult proposition due to the counteracting force of radiation damping (Abragam, 1961; Guéron et al., 1991; Vlassenbroek et al., 1995; Augustine, 2002). The radiation damping field created by transverse water magnetization increases with the static magnetic field and the quality factor of the probehead. In order to rotate the water magnetization by on-resonance irradiation, the radiofrequency (rf) power needs to overcome the radiation damping field. Using a room-temperature triple-resonance, triple-axes gradient probehead on our 800 MHz NMR spectrometer, preirradiation field strengths of 75 Hz can be required to overcome the radiation damping field created by a dilute peptide sample in 90% H_2O/10% D_2O and obtain adequate water suppression. Clearly, such a strong preirradiation field causes considerable saturation of H^{α} resonances and, hence, bleaching in homonuclear 2D NMR spectra (Wider et al., 1983). The problem becomes correspondingly more severe on cryogenic probes.

Due to the vagaries of water suppression by selective pre-irradiation, the much more robust Watergate scheme (Piotto et al., 1992; Sklenář et al., 1993) has become very popular. Unfortunately, it fails as a water suppression scheme in COSY (Aue et al., 1976) and DQF-COSY (Piantini et al., 1982) experiments, because the basic

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phase cycles of these experiments occasionally invert the water magnetization. Inverted water magnetization presents an unstable situation, where a minor disturbance creating some transverse magnetization triggers the return of the water magnetization to its equilibrium position along the positive z-axis by radiation damping, with fully transverse magnetization on the way (Otting and Liepinsh, 1995a; Augustine, 2002). In probeheads with high Q-factor this happens quickly and during the acquisition time of the COSY experiments. Although radiation damping during acquisition can, in principle, be suppressed by Q-switching (Maas et al., 1995), bipolar gradients applied between individual sampling points (Zhang and Gorenstein, 1996), addition of glycine in high concentrations (Rodriguez et al., 2002) or a feedback-loop to counteract radiation damping (Broekert and Jeneer, 1995; Louis-Joseph et al., 1995), all these schemes either reduce sensitivity, require special sample conditions or depend on specialized hardware that is not widely available. Furthermore, the non-uniform excitation profile of Watergate would affect any H^{α}–H^{N} COSY cross-peak for which the H^{α} spin is not rotated by a multiple of 180° during the Watergate sequence. DQF-COSY spectra recorded with gradient selection during the double-quantum filter deliver outstanding water suppression (Hurd, 1990; Davis et al., 1991; John et al., 1992; van Zijl et al., 1995), but at the price of up to 4-fold reduced sensitivity compared with conventional COSY spectra. A similar reduction in sensitivity is observed in ZZCOSY (Zuiderweg, 1987).

Here we propose a variant of the WET scheme (Ogg et al., 1994; Smallcombe et al., 1995) as a generally applicable strategy to replace water saturation by selective preirradiation. The original WET scheme is based on a series of water-selective excitation pulses followed by pulsed field gradients to defocus the transverse water magnetization. On systems with high Q-factor, water-selective pulses are difficult to apply and they must be sufficiently intense to overcome radiation damping (Otting, 1997; Cutting et al., 2000). In our approach, each selective pulse is broken up in the DANTE fashion, inserting bipolar pulsed-field gradients in the delays (Böckmann and Guittet, 1996). The resulting scheme, dubbed secure WET (SWET), provides reliable water suppression with much less power than selective water preirradiation. In addition, the Bloch-Siegert shifts (Ramsey, 1955) resulting from water irradiation during the evolution time of COSY experiments (Wider et al., 1983) were assessed and the water suppression mechanism behind conventional water preirradiation was investigated.

Materials and methods

Experiments were performed on a Bruker Avance 800 MHz NMR spectrometer equipped with a triple-resonance ($^{1}H/^{13}C/^{15}N$) probe operating at room-temperature. Measurements were performed at 25 °C using a 3.5 mM sample of hen egg-white lysozyme in 90% H_2O/10% D_2O at pH 7.0 and a 100 μM sample of C-peptide (31 residues, Ohtomo et al., 1998) in 90% H_2O/10% D_2O at pH 6.9, using conventional 5 mm NMR tubes. In all experiments, the carrier frequency was at the water frequency. The radiation damping field strength was determined by fitting the FID observed after a 90° pulse with the equation (Mao et al., 1994)

$$M_y(t) = M_0 \,\mathrm{sech}(t/T_\mathrm{r}) \qquad (1)$$

where M_y is the magnetization along the y axis, M_0 the equilibrium magnetization, T_r the radiation damping time constant, and t the observation time of the FID. Measurements of the signal-to-noise ratio as a function of receiver gain, using the standard ethylbenzene sample in chloroform, showed that the sensitivity was invariant for receiver gain settings above 512, with about 15% loss in sensitivity for a receiver gain of 128. A receiver gain of 128 was subsequently deemed acceptable. Nutation experiments were performed by pulsing during the acquisition in homogated decoupling mode with a duty cycle of 20%. COSY spectra were baseline corrected in the spectral region $\delta_1 = 1.0$–6.5 ppm/$\delta_2 = 5.5$–10.0 ppm by subtracting 5th order polynomials in both dimensions in order to remove dispersive tails from the diagonal peaks.

Results

Radiation damping field strength

The rate of rotation ω_rd of the water magnetization due to the radiation damping field depends on the

angle θ between the water magnetization and the main magnetic field (the *z*-axis) (Abragam, 1961)

$$\omega_{rd} = \frac{-\sin\theta}{T_r} \quad (2)$$

On our spectrometer, fitting of the FID observed for a 90% H_2O/10% D_2O sample after a 90° pulse (Equation 1) yielded a T_r value of 4.3 ms, corresponding to a rotational frequency $\omega_{rd}/2\pi$ of about 37 Hz for fully transverse water magnetization. Any selective water irradiation scheme must be applied with a higher field strength to overcome the radiation damping. For field strengths little above 37 Hz, radiation damping will significantly slow down the overall rate of rotation.

Conventional water preirradiation

Conventional water saturation by preirradiation typically uses about 1 s of selective irradiation before the first pulse of the experiment, combined with at least two dummy scans to achieve a steady state. A nutation experiment performed with data acquisition in homo-gated decoupling mode showed that the water signal was strongly attenuated after about 40 rotations (Figure 1). This attenuation was achieved in a time much shorter than the T_1 and T_2 relaxation times of the water which are about 2 s (Denisov and Halle, 2002). The same number of rotations produced a very similar attenuation also when much higher power levels were used, confirming that the decay is due to rf-inhomogeneity. Accordingly, a 2 ms trim-pulse applied at a rf-field strength of 20000 Hz provides good water suppression and is better than a 1 ms trim-pulse (Otting and Wüthrich, 1988; Otting, 1994). If relaxation effects can be neglected, Fourier transformation of the nutation data yields the rf-frequency distribution that reflects the rf-inhomogeneity. Figure 1b shows that the frequency distribution is narrow but of finite width. The width of the signals scaled with increasing nutation frequency, as expected for negligible effects from water relaxation (data not shown). The splitting of the peaks corresponds to a difference in nutation frequency of no more than 1.1%. The peaks are asymmetric with long tails of weak intensity towards zero frequency.

In principle, 40 nutations within a recovery delay of 1 s require a water-irradiation field strength of 40 Hz. In the presence of radiation damping, however, the field has to be higher, because the presence of a radiation damping field of up to 37 Hz slows down the overall nutation frequency according to

$$\frac{d\theta}{dt} = \omega_{rf} + \omega_{rd} = \omega_{rf} - \frac{\sin\theta}{T_r} \quad (3)$$

Integration between 0 and 2π for $\omega_{rf}/2\pi = 40$ Hz and $T_r = 4.3$ ms yields an average nutation frequency of only 18 Hz.

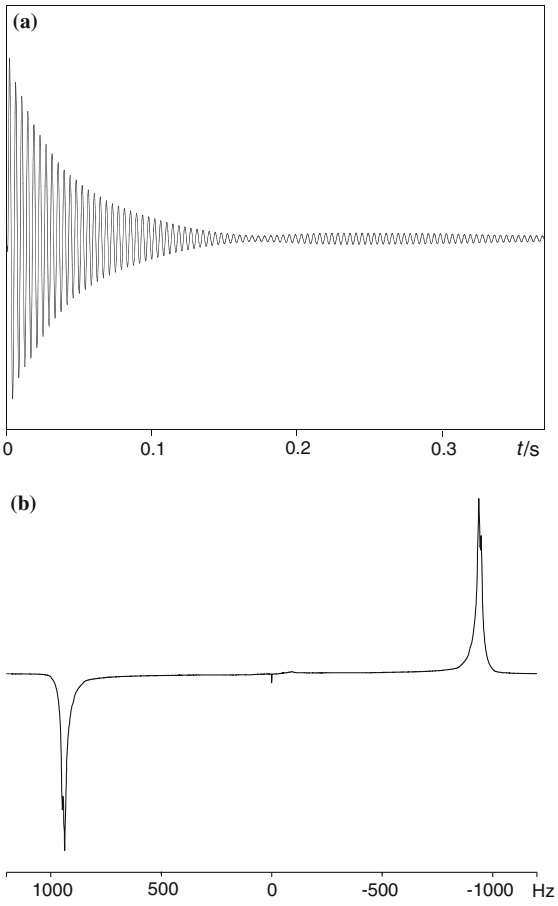

Figure 1. Nutation experiment for the measurement of rf-inhomogeneity. The data were acquired as a single FID while the ^1H NMR signal of a sample of 90% H_2O/10% D_2O was irradiated with homogated decoupling. (a) Real part of the FID. There was almost no signal in the imaginary part. (b) Fourier transform of the FID in (a), showing the distribution of nutation frequencies in the experiment.

Bilevel water irradiation

The necessity of at least 40 nutations for optimal water suppression by preirradiation combined

with reduced radiation damping once the water magnetization is partially defocused suggests that improved selectivity could be obtained by division of the preirradiation period into an initial period of increased irradiation power to minimize the radiation damping effects followed by a period with weaker irradiation to complete the number of nutations while allowing recovery of the magnetization of solute signals near the water resonance. We found that such a bilevel irradiation scheme worked adequately for a concentrated lysozyme sample, reducing the number of H^{α} signals bleached by the preirradiation. However, unacceptably high preirradiation powers were still required to record spectra with our dilute peptide sample.

Swet

The WET scheme (Ogg et al., 1994; Smallcombe et al., 1995) provides good water suppression independent of sample concentration, but radiation damping on probeheads with high Q factor limits its selectivity. This drawback is overcome by the SWET scheme (Figure 2), where each of the selective water pulses is delivered as a train of small flip-angle pulses interleaved with bipolar gradients as in WANTED-type water excitation

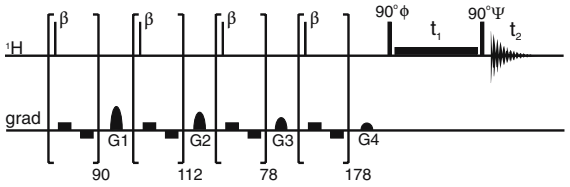

Figure 2. COSY pulse sequence preceded by SWET. Typical parameters for the SWET scheme are: flip-angle $\beta \approx 1°$ with 1.4 μs duration; the flip-angle must be interactively fine-tuned for maximum water suppression; amplitude of the bipolar gradients: 10 G/cm, with each pulsed field gradient of rectangular shape of 50 μs duration and followed by a 50 μs recovery delay; gradient pulses G_1–G_4: sine shaped of 1 ms duration each and with amplitudes of 32, 16, 8 and 4 G/cm, respectively; each gradient pulse was followed by a 100 μs recovery delay. Instead of bipolar gradients, a Q-switch could be used to suppress radiation damping (Otting and Liepinsh, 1995b). The pulse spacing must be less than twice the dwell time to avoid excitation sidebands within the spectral width. Continuous irradiation on the proton channel at a power level of 15 Hz during the t_1 evolution period of the COSY pulse sequence serves to maintain the water suppression for long t_1 values. Phase cycle: ϕ = x,x,−x,−x; ψ = x,−x; receiver = x,x,−x,−x, with quadrature detection achieved by States-TPPI.

(Böckmann and Guittet, 1996). WANTED pulses suppress radiation damping and can be made highly selective. In our hands, rectangular WANTED pulses with an average field strength of 15 Hz provided adequate water suppression in the SWET scheme. This value, found by experimental optimization for best water suppression, was somewhat higher than predicted from the nominal flip angles of the SWET sequence which could be explained by finite rise and decay times of the pulses and by incomplete suppression of radiation damping by the bipolar gradients; doubling the duration of the SWET pulses by doubling the number of pulse-delay elements yielded adequate water suppression only if the pulse amplitudes were reduced by less than 50%. The flip-angles proposed for the WET sequence optimized for T_1 and rf imperfections (Ogg et al., 1994) yielded the best water suppression with SWET. One-dimensional NMR spectra recorded of C-peptide with water suppression by SWET or conventional selective water irradiation with significantly increased power resulted in residual water signals of comparable size. While the intensities of the aliphatic signals of the peptide were indistinguishable, the signals of the exchangeable amide protons were more intense with SWET due to reduced saturation transfer, since SWET took up only the last 96 ms of the recovery delay between scans (Figure 3).

Since 2D NMR experiments are invariably recorded with recycling delays that allow only incomplete relaxation of the water magnetization, the remaining steady-state magnetization is less prone to radiation damping. In the case of the dilute solution of C-peptide on our 800 MHz NMR spectrometer, the steady-state water magnetization was at least 15% of the equilibrium water magnetization after recovery during 0.5 s of data acquisition and the water magnetization had grown to about 50% of its equilibrium value after 1.5 s. In this situation, a conventional WET scheme containing four Seduce-shaped pulses of 20 ms duration each (Smallcombe et al., 1995) did no longer yield adequate water suppression. In principle, the WET sequence could be implemented by replacing the first selective WET pulse by a radiation-damping compensated shaped pulse (Chen et al., 1999; Cutting et al., 2000), but this would require that the shape of the pulse is adjusted to the steady-state magnetization of the

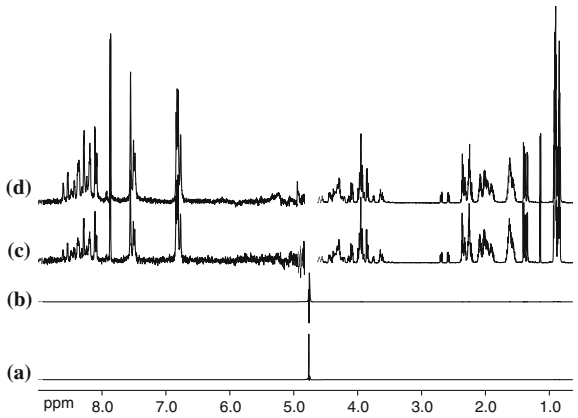

Figure 3. 1D NMR spectra recorded of a 100 μM solution of C-peptide in 90% H_2O/10% D_2O at pH 6.9 and 25 °C, using water suppression by 1 s of preirradiation with an amplitude of 75 Hz (a and c) or SWET with a total duration of 96 ms using an average pulse amplitude of 15 Hz (b and d), followed by a hard 90° pulse. Both spectra were recorded with an acquisition time of 0.51 s, using 128 scans, eight dummy scans and the same total recycling delay. No postacquisition processing was used to suppress the water resonance in (a) and (b), whereas the baseline was corrected manually near the water resonance in (c) and (d). (a) Residual water signal in the spectrum recorded with preirradiation. (b) Residual water signal in the spectrum recorded with SWET plotted on the same scale as (a). (c) Solute signals in the spectrum recorded with preirradiation. The vertical scale was expanded 1000-fold in the aliphatic region and 16700-fold in the amide region compared to the spectra in (a) and (b). (d) Solute signals in the spectrum recorded with SWET, plotted with the same magnification as in (c). SWET resulted in virtually identical signal intensities in the aliphatic region of the spectrum and less signal attenuation of the amide protons.

water present at the start of the WET sequence which in turn depends on the acquisition time and recycling delay used. Adequate water suppression was possible, when the shape of the first WET pulse was kept unchanged but its duration was shortened to 5 ms. This, however, would have resulted in substantial saturation of a wide band of H^{α} resonances. SWET combines the advantages of a simple setup with selectivity of water suppression.

Water suppression in COSY

Although the SWET scheme provided adequate water suppression in 1D and 2D NMR experiments recorded with short evolution times, the water suppression deteriorated significantly during long t_1 evolution times of COSY and DQF-COSY experiments due to the recovery of equilibrium water magnetization. This problem was solved by application of weak selective water irradiation during t_1 (Figure 2). A rf-irradiation strength of 15 Hz (i.e., of similar average power as the SWET pulses) was sufficient to maintain adequate water suppression throughout the 2D experiments. The scheme of Figure 2 was used to record a COSY spectrum of a 100 μM solution of C-peptide (Figure 4). For consistent water suppression throughout the 2D experiment, the pulse power used for SWET was optimized using a one-dimensional experiment based on the pulse sequence SWET – 90°-pulse – acquisition. Baseline corrections in both dimensions removed the dispersive tails from the diagonal peaks, yielding a perfectly flat baseline. Conventional water preirradiation during 1 s combined with the same acquisition time (293 ms) required the use of a 75 Hz irradiation field to allow the same receiver gain setting and selective water irradiation during t_1 would still have been necessary to maintain the water suppression for long t_1 values.

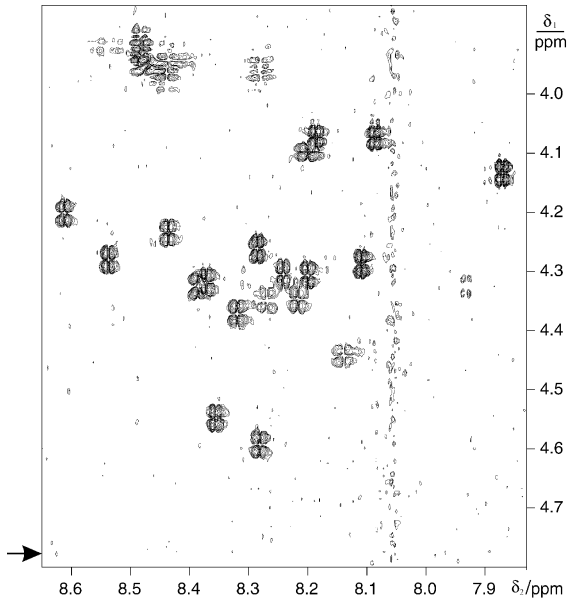

Figure 4. Fingerprint region of a COSY spectrum recorded with the pulse scheme of Figure 2, using a 100 μM sample of C-peptide in 90% H_2O/10% D_2O at pH 6.9 and 25 °C. The arrow identifies the frequency of the water resonance. The spectrum was recorded with $t_{1max} = 100$ ms and $t_{2max} = 293$ ms, using 28 scans per FID, spectral widths of 7000 Hz in both dimensions, a total recycling delay of 1 s (excluding the acquisition time but including the SWET duration of 96 ms) and a total experimental time of 16 h.

The shorter duration and the lower power needed for SWET compared to water suppression by selective preirradiation translates into reduced saturation of H^{α} resonances near the chemical shift of the water. This effect was experimentally verified by COSY spectra recorded of hen egg-white lysozyme using, respectively, a 96 ms SWET sequence at 15 Hz average power or water preirradiation with 40 Hz field strength during 1 s, respectively (Supplementary material).

Bloch-Siegert shifts

Water irradiation during evolution periods have been shown to result in frequency shifts of signals near the irradiation frequency (Wider et al., 1983). Since they are based on the same mechanism as frequency shifts due to the counter-rotating field contained in a linearly polarized radio-frequency field (Ramsey, 1955), we refer to these shifts in the following as Bloch-Siegert shifts. For the small irradiation power used in our experiments, however, these frequency shifts are small and would hardly interfere with the analysis of protein NMR spectra. Since the C-peptide has no signals near the water resonance, this effect was studied by comparison of a COSY spectrum of hen egg-white lysozyme recorded with 15 Hz water irradiation during the t_1 evolution with a TOCSY spectrum recorded without water irradiation during the evolution period (Figure 5). Except for the cross-peak of Arg68 which was very close to the water chemical shift at 4.765 ppm, no significant frequency shifts were evident in the indirect dimension of the COSY spectrum. Frequency shifts due to heating effects by the TOCSY mixing period were at least as pronounced as the frequency shifts induced by the water irradiation during the t_1 period of the COSY experiment.

Any Bloch-Siegert shifts of H^{α}–H^{N} cross-peaks in spectra recorded with 30 Hz irradiation amplitude were still smaller than the widths of the H^{α} multiplets (Figure 5b). The small magnitude of the frequency shifts to be expected from water irradiation during the evolution time is confirmed by the quantitative prediction presented in Figure 6. For example, a frequency shift of less than 3 Hz is predicted for any cross-peak further than 30 Hz from the water resonance, if the irradiation amplitude is 15 Hz.

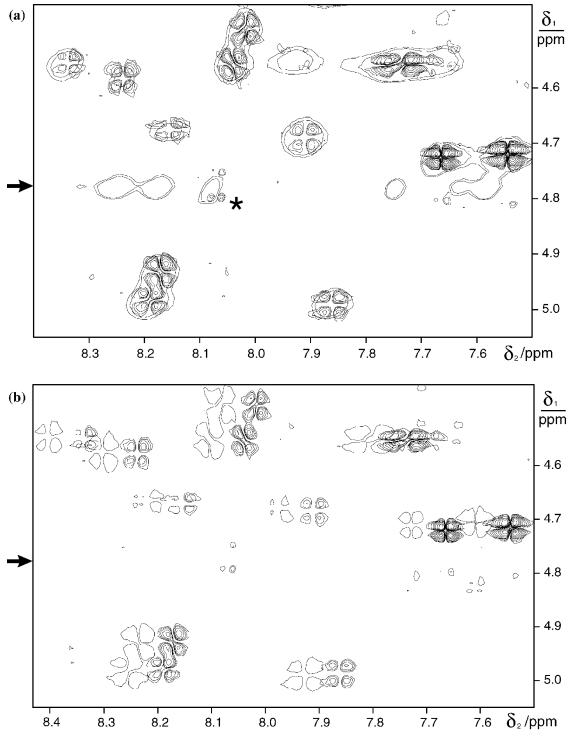

Figure 5. Frequency shifts due to water irradiation during the evolution time. Selected spectral region from the fingerprint region of homonuclear COSY and TOCSY spectra recorded with a 3.5 mM solution of hen egg-white lysozyme in 90% H_2O/10%D_2O at pH 7.0 and 25 °C. The arrow identifies the frequency of the water resonance. (a) Superposition of a COSY spectrum recorded with continuous irradiation at 15 Hz during the t_1 evolution period with a TOCSY spectrum recorded without water irradiation during t_1. The TOCSY spectrum contains additional cross-peaks at the water frequency due to chemical exchange. Only the two lowest contour lines are shown for the TOCSY spectrum. Water suppression in the TOCSY experiment was achieved by weak preirradiation during the recycle delay and a Watergate sequence following the mixing period. The H^{α}–H^{N} cross-peak of Arg68 is identified by a star. (b) Superposition of the spectral regions from two COSY spectra recorded using irradiation during t_1 evolution with amplitudes of 30 and 15 Hz, respectively. The spectrum with 15 Hz irradiation was plotted on a logarithmic scale with a factor of 1.4 between subsequent contour levels. Only the lowest contour level is shown for the COSY spectrum recorded with irradiation strength of 30 Hz. The latter spectrum was shifted horizontally to facilitate comparison with the COSY spectrum recorded with 15 Hz irradiation.

Discussion and conclusions

H^{α}–H^{N} COSY cross-peaks contain valuable information for the resonance assignment of unlabelled proteins (Wüthrich, 1986). Since COSY spectra have a high intrinsic sensitivity, some

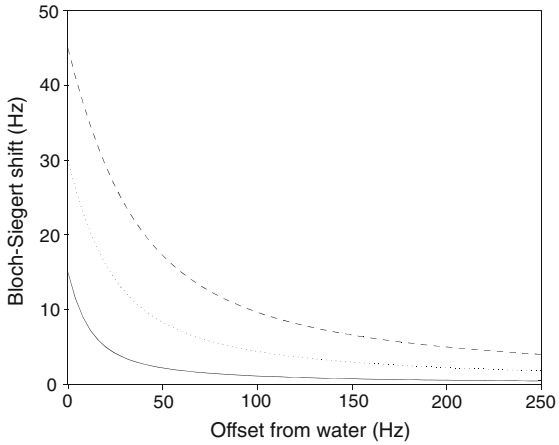

Figure 6. Prediction of the frequency shift of a protein spin as a function of its offset from the water irradiation frequency. The curves were calculated for water irradiation amplitudes of 45 (— —), 30 (– – –) and 15 Hz (———), respectively, using the relation $\omega_{BS} = \sqrt{\Omega^2 + \omega_1^2} - \Omega$, where ω_{BS} is the frequency shift, Ω is the offset of the Larmor frequency of the spin from the water frequency, and ω_1 is the amplitude of the water irradiation field. The graph approximates the relation $\omega_{BS} = \frac{\omega_1^2}{2\Omega}$ for $\Omega \gg \omega_1$ (Ramsey, 1955).

attenuation of the protein signals by saturation transfer is often acceptable. For the spectral region containing the H^{α}–H^{N} cross-peaks, the dispersive tails of the diagonal peaks can readily be removed by baseline correction. The present study was prompted by the difficulty to record COSY spectra with conventional water suppression by selective preirradiation.

On NMR spectrometers 30 years ago, water suppression by selective irradiation relied on rf-inhomogeneity (Hoult, 1976) more than on the interplay of T_1 and T_2 relaxation (Torrey, 1949). Our present data show that this situation still holds today for the short irradiation times usually used in 2D NMR experiments. Therefore, the spatial scrambling of the water magnetization achieved by the WET or SWET schemes is conceptually not different from the spatial scrambling resulting from selective irradiation. In this situation, a WET scheme appears superior since the irradiation is shorter, giving rise to less saturation of solute resonances. For concentrated protein solutions and in the presence of compounds undergoing proton exchange with the water, the water resonance is broadened by the chemical exchange. In this case, conventional water irradiation can yield better water suppression than SWET because the effective T_2 relaxation time of the water resonance is shortened by the exchange-broadening of the water, adding saturation of the water resonance (Torrey, 1949) as a significant mechanism of water suppression. With the advent of increasingly more sensitive NMR spectrometers, however, ever more dilute samples are being investigated, where the water resonance is little affected by the solute. Particularly under those circumstances, SWET provides superior water suppression compared with the conventional water preirradiation scheme by allowing the use of the same receiver gain setting with less power applied for a shorter time period resulting in reduced saturation of protein resonances.

Acknowledgements

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Supplementary material is available comparing the selectivity profiles achieved by SWET and 1 s water irradiation in (i) simulations and (ii) experimental COSY spectra showing the saturation of H^{α} resonances. It is available in electronic format at: http://dx.doi.org/10.1007/s10858-005-8531-6.

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