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Use of a water flip-back pulse in the homonuclear NOESY experiment

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Summary

A simple modification to the WATERGATE water suppression scheme [Piotto, M., Saudek, V. and Sklenář, V. (1992) *J. Biomol. NMR*, **2**, 661–665] is proposed. Radiation damping is used as an active element during the mixing time of a NOESY experiment, in order to obtain a reproducable state of the water magnetization at the end of the mixing time. Through the use of a water flip-back pulse and a gradient-tailored excitation scheme, we obtain both an excellent water suppression and a water magnetization close to equilibrium at the beginning of the acquisition time. We show experimentally that this modification results in a 20% gain in intensity for all signals when using a relaxation delay of 1.5 s, and also that avoiding a semisaturated state for the water magnetization allows the amide protons as well as other proton resonances to relax to equilibrium with their proper relaxation time.

Most NMR studies of biological macromolecules require the recording of different spectra in aqueous solution, and hence suffer from the problem that the proton concentration is not equally distributed between the 110 M concentration of the water protons and the millimolar concentration of the protons of the molecule under study. A number of selective elimination schemes have been developed, based on the selective presaturation of the water resonance (Hoult, 1976; Hore, 1989), on the use of spin-lock pulses (Messerle et al., 1989; Sodano and Delepierre, 1993), on a selective zero-excitation of the water resonance using jump-return sequences (Plateau and Guéron, 1982; Plateau et al., 1983) or shaped pulses (Smallcombe, 1993), on the difference between the relaxation times of the water and protein proton resonances (Brown et al., 1988) and recently methods involving gradient-enhanced pulse sequences have been proposed based on the different diffusion properties of the solvent and solute (Van Zijl and Moonen, 1990) or on selective defocussing of the undesired resonance (Piotto et al., 1992).

The first method of presaturation has some severe drawbacks, especially when hydrogen exchange is rapid, causing a saturation transfer to certain protons of interest as well as to other protons through the mechanism of spin diffusion (Otting et al., 1991; Liepinsh et al., 1992; Moy et al., 1992; Henry and Sykes, 1993; Li and Montelione, 1993; Smallcombe, 1993). The sequences based on the use of jump-return or of selective pulses do not excite the water resonance, thereby effectively eliminating all saturation transfer. However, a recent publication described how the combination of radiation damping effects and the need to achieve ω_1 frequency discrimination can lead to a lesser degree of water suppression than hoped for (Stonehouse et al., 1994). These authors therefore modified the original sequence, including a gradient pulse in order to achieve a constant water suppression for all phases and t_1 increments. The use of gradients, combined with selective excitation of the water line, causes a refocusing of all resonances except the water resonance, thereby reducing the water line in a single scan by four to five orders of magnitude (Piotto et al., 1992).

While the latter spectra retain a pure phase character as well as a perfectly flat baseline, the water resonance often remains in a semisaturated state, as for practical reasons the relaxation delay between scans is much shorter than the water T_1 (4–5 s). Several 'water flip-back' sequences have been described (Grzesiek and Bax, 1993a,b; Jahnke and Kessler, 1994; Kuboniwa et al., 1994) in the context of sensitivity enhancement of heteronuclear spectra, where the intensities of the ^1H-^{15}N correlations suffer

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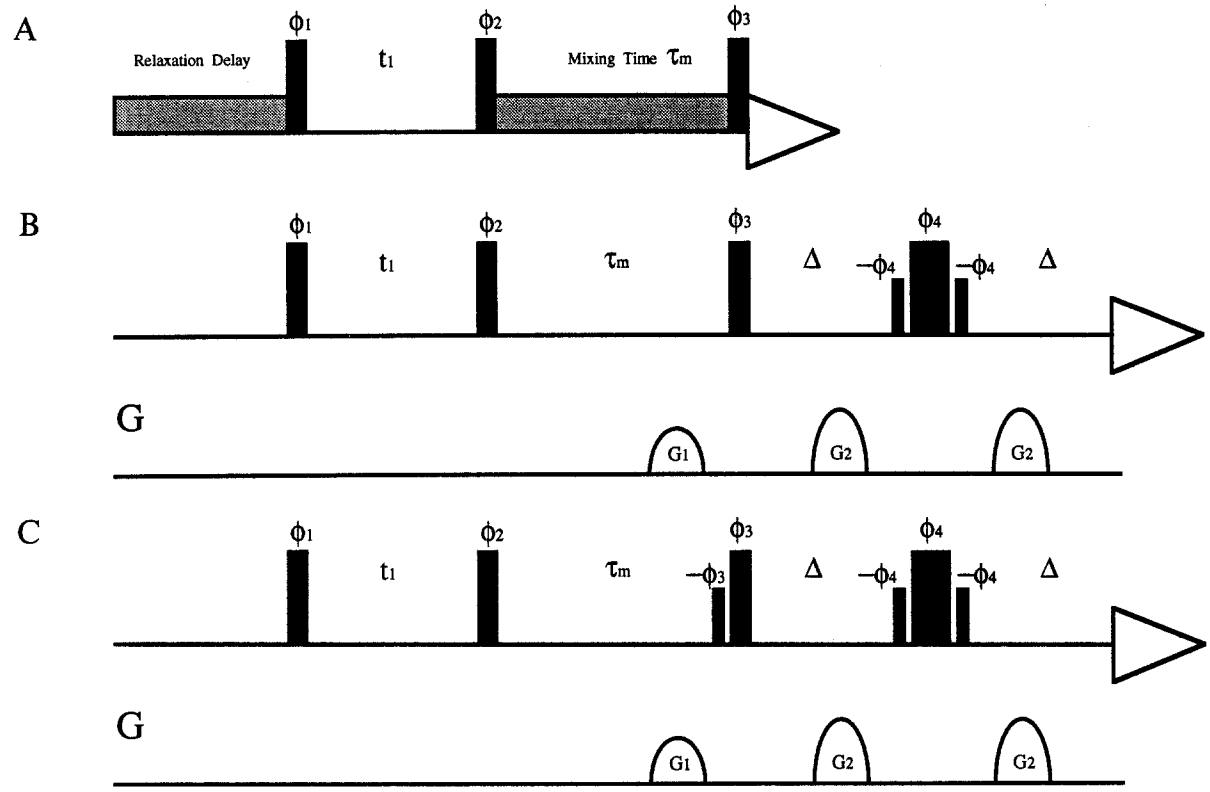

Fig. 1. Pulse sequences of (A) the classic NOESY experiment with low-power presaturation (shaded pulses) during the relaxation delay and the mixing time τ_m; (B) the WATERGATE NOESY experiment including a gradient G_1 at the end of the mixing time and the gradient-tailored excitation scheme (Piotto et al., 1992); and (C) the water flip-back WATERGATE NOESY experiment, including a selective pulse at the end of the mixing time. Phases used are $\phi1$ = (x,–x,y,–y,–x,x,–y,y), $\phi2$ = (x,x,y,y,–x,–x,–y,–y), $\phi3$ = (x,x,y,y,–x,–x,–y,–y), $\phi4$ = (–x,–x,–y,–y,x,x,y,y) and Acq. = (x,–x,y,–y,–x,x,–y,y).

heavily from any form of saturation transfer. However, also in the homonuclear NOESY spectrum (Kumar et al., 1980) rapid to intermediate exchange of the amide protons with the semisaturated water magnetization will lead to a decrease in intensity, especially for such important cross peaks as the NH-H^{α} correlations. In this communication, we demonstrate that the combination of a water flip-back selective pulse with a water suppression scheme such as the WATERGATE sequence (Piotto et al., 1992) can be used to obtain both a good water suppression and a state of the water magnetization close to equilibrium at the beginning of the acquisition time. Moreover, we will show that the repetition rate in this sequence is solely determined by the T_1 relaxation times of the protein magnetization.

The basic NOESY sequence is shown in Fig. 1A. Water presaturation is applied during both the relaxation delay and the mixing time, leading to a net water magnetization close to zero at the beginning of the acquisition period. The water magnetization relaxes back to its equilibrium value (parallel to the z-axis) during the acquisition time, but the B_1 field during the relaxation delay reduces it back to zero. The resulting signal of a single-scan experiment (where, starting from equilibrium, one probes the water magnetization along the z-axis with a 90°_{x} pulse during the acquisition time) shows that water is basically reduced to zero at the beginning of the acquisition time, and relaxes back to its equilibrium value through the mechanism of T_1 relaxation.

In the WATERGATE sequence (Fig. 1B), no presaturation is applied at all, and one has to take into account the phenomenon of radiation damping (Bloembergen and Pound, 1954; Warren et al., 1989; Vlassenbroek, 1993) during the mixing period: depending on the phase cycle, the water magnetization will start along the –z-axis or in the xy-plane, but in both cases, the return of the magnetization to the +z-axis will be much faster than predicted by the relaxation equations. For a 600 MHz spectrometer equipped with a well-tuned high-Q factor probehead, the characteristic times of return to the +z-axis are as short as 50 ms, even after an optimized 180° pulse (Vlassenbroek, 1993; Stonehouse et al., 1994). Therefore, at the end of the mixing time the water vector invariantly points to the +z-axis, irrespective of the t_1 increment or the moment in the phase cycle. The small gradient pulse at the end of the mixing time does not affect the water magnetization along the +z-axis, but assures that transverse coherences of the protein magnetization that otherwise need to be cancelled by phase cycling are eliminated in a single scan. The water vector along the +z-axis is then brought into the

xy-plane by the third 90° pulse, after which it is effectively scrambled by the two gradient pulses. The latter act constructively only for a small region around the water resonance, but allow for refocusing of the other resonances due to the echo that is formed by the central 180° pulse. Even though the water suppression of this scheme is excellent, the net water magnetization vector is zero at the beginning of the sequence, and water will return to equilibrium during the acquisition and relaxation delays. Its return is somewhat more rapid than in cases where presaturation is used, which can be attributed to the eventual refocussing of a fraction of the transverse water component, followed by a small amount of radiation damping (M. Piotto, personal communication). However, this return still takes on the order of seconds, and as for considerations of total measuring time the combined duration of acquisition and relaxation delay is usually kept as short as possible, the result is a 'semisaturated' state of the water magnetization.

Radiation damping during the mixing time can be used advantageously when it is recognized that the state of the water magnetization at the end of the mixing time is perfectly reproducible, which is exactly what we hope to have: all water magnetization is back to its equilibrium state along the +z-axis. If the final 90° pulse is now preceded by a selective pulse of opposite phase (Fig. 1C), the water magnetization after the third 90° pulse will still be along the +z-axis, and the subsequent gradients and 90°-($-\phi_4$)–180°(ϕ_4)–90°($-\phi_4$) will not affect this magnetization at all. The result is that the acquisition time is started without any transverse water component, but with a longitudinal water component very close to its thermal equilibrium and thus invariant in time. Radiation damping should thus be considered as an active element in this pulse sequence to obtain the desired water magnetization state. This experimental scheme avoids the development of a semisaturated water state and its possible transfer to the protein resonances, and the T_1 of the protein resonances becomes the limiting factor for the choice of the relaxation delay.

For short mixing times, the statements given above are not completely valid, but the gradient at the end of the mixing time does ensure that the only component of the water magnetization that remains just before the third 90° pulse is oriented along the +z-axis. As the slowest phase of radiation damping is the initial buildup of a macro-

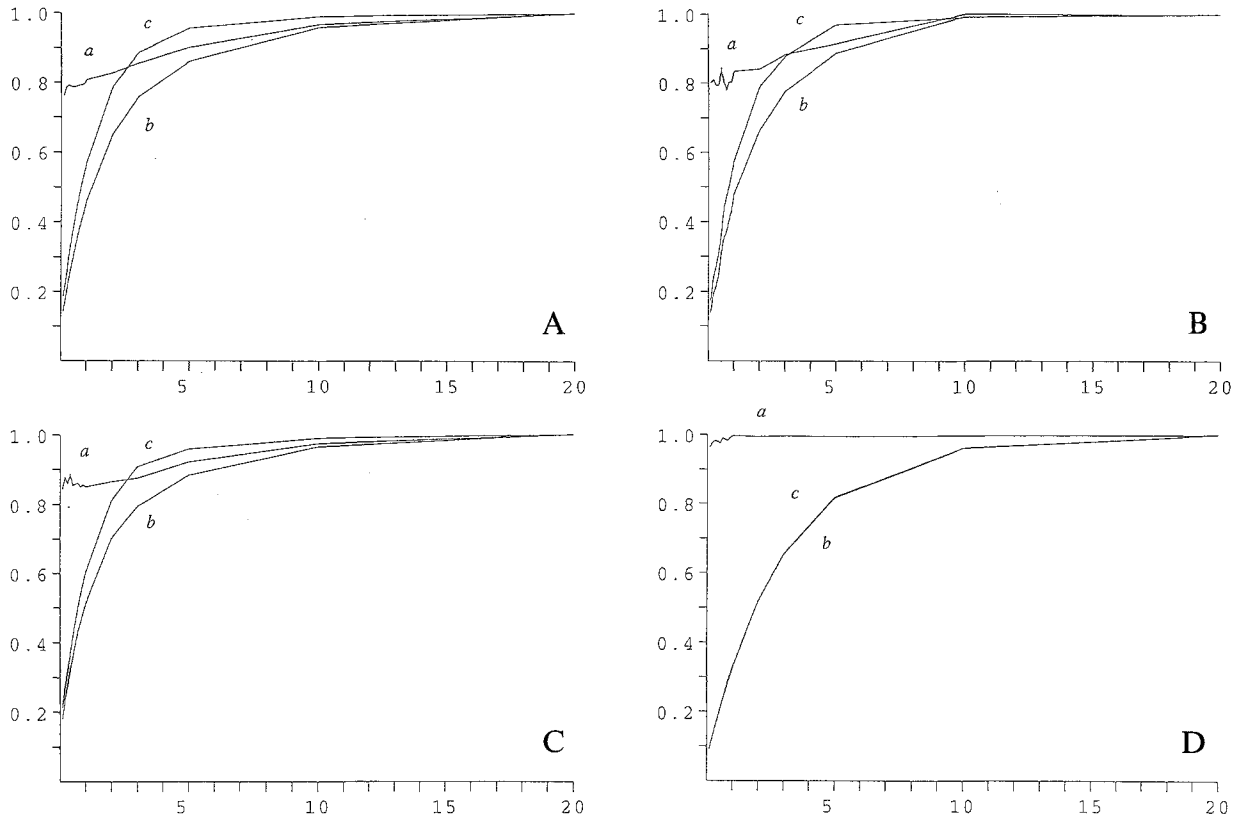

Fig. 2. Intensities of selected resonances in the 1D spectra obtained with the pulse sequences of Fig. 1B (indicated by *b*) and Fig. 1C (indicated by *c*) as a function of the relaxation delay between successive scans and normalized with respect to the intensity obtained with a relaxation delay of 20 s. The ratio *b*/*c* is indicated by *a*. Resonances are (A) the NH proton at 7.16 ppm; (B) the α resonance at 5.87 ppm; (C) the methyl resonance at –0.64 ppm; and (D) the TMSP resonance at 0 ppm. The experimental conditions are the same as in the legend of Fig. 3. Apparent T_1 values were obtained by fitting an exponential to the intensities obtained at different relaxation delays using the program SNARF (F. van Hoesel, University of Groningen, Groningen, The Netherlands).

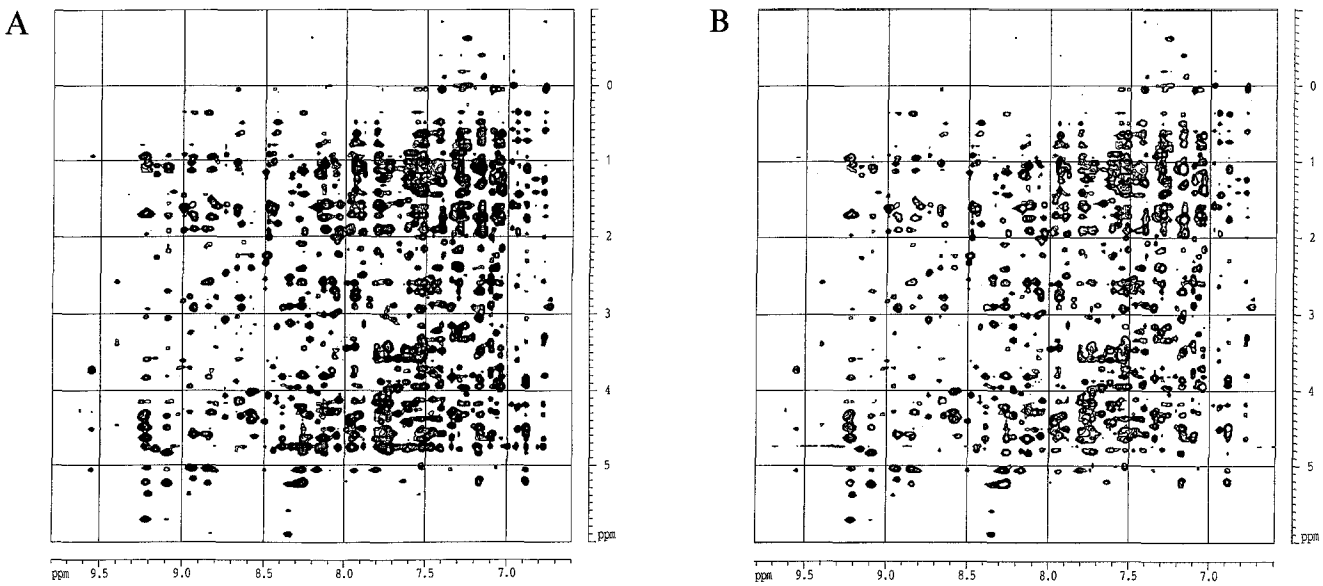

Fig. 3. Phase-sensitive 2D NOESY spectrum of hen egg white lysozyme. Sample conditions : pH 6.7, 30 µl of a 1 M TMSP solution, and a 2 mM solution of lysozyme in 500 µl of a 90% H_2O/10% D_2O solution, T = 35 °C. Spectra were obtained on a Bruker 600 MHz DMX spectrometer operating at 600 MHz and equipped with a triple-resonance self-shielded z-gradient probehead. Gradients have a sine-bell shape, with maximum gradient strengths of 5 G/cm for the gradient in the mixing time (G_1, Figs. 1B and C) and 15 G/cm for the gradients in the WATERGATE suppression sequence (G_2, Figs. 1B and C). Selective pulses correspond to a field strength of 160 Hz. The mixing time was 300 ms. Acquisition parameters: spectral width of 14 ppm in both dimensions, 2K complex points in t_2 and 256 complex t_1 increments (States et al., 1982), with 8 scans per increment. (A) 2D spectrum obtained with the pulse sequence of Fig. 1C and a mixing time of 300 ms. (B) Difference spectrum between the 2D spectra obtained with the sequences of Figs. 1C and B. The threshold for spectrum B is a factor of two lower than that for A.

scopic transversal component of the water magnetization, one can enhance its rate either by using a second pulse that is slightly shorter than the real 90° pulse (M. Piotto, personal communication) or by changing the relative phase between the first and second pulses (Zhang et al., 1994). In this manner, an equilibrium magnetization along the +z-axis can be obtained with a mixing time as short as 40 ms, with only a minor loss in signal intensity.

As previous studies have extensively compared the effect of presaturation on the resonance intensities (Smallcombe, 1993), we will only compare spectra recorded with the sequences shown in Figs. 1B and C. We first measured the efficiency of the water flip-back procedure by comparing the water magnetization at the start of the acquisition time of our NOESY sequence to the equilibrium water magnetization (as measured by a single pulse experiment). When carefully optimizing the flip-back pulse (so as to minimize the net flip angle of the combined $90°(-\phi_3)$ soft pulse–$90°(\phi_3)$ hard pulse), we obtained 75% of the full water magnetization at the start of the acquisition time when using a mixing time of 150 ms. The return of the protein signals towards equilibrium can be monitored by recording a number of spectra with relaxation delays varying from 20 s to a value as small as 0.1 s. The results are shown in Figs. 2A–D: as the relaxation delay becomes shorter, the total intensity of the different resonances decreases, but it does so more rapidly when using the sequence of Fig. 1B than the one of Fig. 1C. When we characterize the decrease by an apparent T_1 value, we find values of 2.2 and 1.4 s for amide protons, 2.1 and 1.4 s for α protons and 2.0 and 1.3 s for methyl resonances, where the first value is for the sequence of Fig. 1B and the second for the sequence of Fig. 1C. As a control, Fig. 2D shows the evolution of the TMSP resonance, which is characterized by an identical T_1 value of 3.0 s for both spectra. The uniform values for the relaxation rates of the different protons in the experiment of Fig. 1C can be understood as the effect of spin diffusion (Kalk and Berendsen, 1976), and are compatible with the values we expect for a protein the size of lysozyme. In the experiment of Fig. 1B, however, the buildup of a semi-saturated state, even in the absence of any presaturation, combined with rapid chemical exchange of some amide protons with water protons due to the high pH (6.7) of the sample, yields apparent T_1 values that are somewhere between those of the protein protons and that of the water magnetization. The effect is of equal magnitude for amide and α protons, and slightly weaker for methyl protons.

The 2D performance of sequence 1C is shown in Fig. 3A. The relaxation delay was set to 1.5 s, which is a typical value for experiments in our laboratory. The water suppression is excellent, due to the gradient-tailored WATERGATE excitation. From Fig. 2, we expect that the intensities involving NH and/or α protons will be smaller by approximately 20% when omitting the selective 90° water flip-back pulse. Figure 3B shows the difference spectrum between the spectrum of Fig. 3A and the spec-

trum obtained by the regular WATERGATE sequence. Both spectra were recorded under identical conditions, resulting in almost identical intensities of the TMSP diagonal peak. All other resonances, however, increased by a factor of 15–25% when using the flip-back pulse during the mixing time.

An aspect of the sequence of Fig. 1C we have not commented upon yet is its robustness and ease of implementation. Indeed, there are very few experimental parameters to adjust, as we used a low-power rectangular pulse identical to those used in the WATERGATE scheme to flip back the water. Any imperfection of the selective pulse in the mixing time will cause the water magnetization to be in a state $(1 - \varepsilon^2)^{1/2}$ of its equilibrium state, where ε is the sine of the net flip angle of the combined selective 90°(–x) and hard 90°(x) pulses. Moreover, as the subsequent water suppression scheme is basically a gradient version of the spin-echo sequence, distortion of the first point is avoided, which eliminates the need of data shifting and/or linear prediction (Davis, 1989). Although the phase of the signals around the water line is not well defined in the ω_2 direction, this is not a problem as the relevant cross peaks are observed in the ω_1 direction. Finally, the modification of the original WATERGATE sequence is almost trivial, and can easily be combined with any sequence where the NOESY is the last step (e.g. HSQC-NOESY) in order to bring the water magnetization close to its equilibrium value at the beginning of the acquisition time, allowing more signal involving rapidly exchanging amide protons to be detected and thus a better definition of the protein structure in regions that would otherwise be considered ill-defined or flexible.

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References

Bloembergen, N. and Pound, R.V. (1954) *Phys. Rev.*, **95**, 8–12.
Brown, S.C., Weber, P.L. and Mueller, L. (1988) *J. Magn. Reson.*, **77**, 166–169.
Davis, D. (1989) *J. Magn. Reson.*, **81**, 603–607.
Grzesiek, S. and Bax, A. (1993a) *J. Am. Chem. Soc.*, **115**, 12593–12594.
Grzesiek, S. and Bax, A. (1993b) *J. Biomol. NMR*, **3**, 627–638.
Henry, G.D. and Sykes, B. (1993) *J. Magn. Reson. Ser. B*, **102**, 193–200.
Hore, P.J. (1989) *Methods Enzymol.*, **176**, 64–77.
Hoult, D.I. (1976) *J. Magn. Reson.*, **21**, 337–347.
Jahnke, W. and Kessler, H. (1994) *J. Biomol. NMR*, **4**, 735–740.
Kalk, A. and Berendsen, H.J. (1976) *J. Magn. Reson.*, **24**, 343–366.
Kuboniwa, H., Grzesiek, S., Delaglio, F. and Bax, A. (1994) *J. Biomol. NMR*, **4**, 871–878.
Kumar, A., Ernst, R.R. and Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun.*, **95**, 1–6.
Li, Y.-C. and Montelione, G.T. (1993) *J. Magn. Reson. Ser. B*, **101**, 315–319.
Liepinsh, E., Otting, G. and Wüthrich, K. (1992) *J. Biomol. NMR*, **2**, 447–465.
Messerle, B.A., Wider, G., Otting, G., Weber, C. and Wüthrich, K. (1989) *J. Magn. Reson.*, **85**, 608–613.
Moy, F.J., Scheraga, H., Patt, S.L. and Montelione, G.T. (1992) *J. Magn. Reson.*, **98**, 451–457.
Otting, G., Liepinsh, E. and Wüthrich, K. (1991) *J. Biomol. NMR*, **1**, 209–215.
Piotto, M., Saudek, V. and Sklenář, V. (1992) *J. Biomol. NMR*, **2**, 661–665.
Plateau, P. and Guéron, M. (1982) *J. Am. Chem. Soc.*, **104**, 7310–7311.
Plateau, P., Dumas, C. and Gueron, M. (1983) *J. Magn. Reson.*, **54**, 46–53.
Smallcombe, S.H. (1993) *J. Am. Chem. Soc.*, **115**, 4776–4785.
Sodano, P. and Delepierre, M. (1993) *J. Biomol. NMR*, **3**, 471–477.
States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982) *J. Magn. Reson.*, **48**, 286–292.
Stonehouse, J., Shaw, G.L. and Keeler, J. (1994) *J. Biomol. NMR*, **4**, 799–805.
Van Zijl, P.C.M. and Moonen, C.T.W. (1990) *J. Magn. Reson.*, **87**, 18–25.
Vlassenbroek, A. (1993) Ph.D. Thesis, Université Libre de Bruxelles, Belgium.
Warren, W.S., Hammes, S.L. and Bates, J.L. (1989) *J. Chem. Phys.*, **91**, 5895–5904.
Zhang, O., Kay, L.E., Olivier, J.P. and Forman-Kay, J.D. (1994) *J. Biomol. NMR*, **4**, 845–858.