JOURNAL OF MAGNETIC RESONANCE **129,** 93–97 (1997)
ARTICLE NO. MN971249

COMMUNICATIONS

ROESY with Water Flip Back for High-Field NMR of Biomolecules

D. Bruce Fulton[1] and Feng Ni[2]

Biomolecular NMR Laboratory and the Montreal Joint Centre for Structural Biology, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2

Received March 20, 1997; revised July 25, 1997

We report a version of the ROESY experiment in which saturation of the water magnetization is avoided without compromising suppression of the water signal during acquisition. Field gradient and selective RF pulses are used to maintain precise control of the water magnetization throughout the experiment and avoid signal losses due to radiation damping and molecular diffusion effects. The pulse sequence includes a delay for intentional radiation damping prior to mixing period. The optimal length of this delay is field and sample dependent, but easily determined from the apparent linewidth of the water signal. NOESY and TOCSY variants of the same experiment are presented which make use of identical manipulations of the water magnetization. The three pulse sequences constitute a suite for which little parameter adjustment is required once one of the experiments has been configured.

In order to obtain maximum sensitivity in high-field ($\nu_0 \geq 500$ MHz) NMR spectroscopy of aqueous samples it is necessary to suppress the water signal during acquisition while avoiding saturation or dephasing of the water magnetization during the majority of the experimental cycle, particularly for samples at neutral pH (*1*). This was first demonstrated in heteronuclear experiments (*2*), and more recently for homonuclear NOESY (*3, 4*) and TOCSY (*5, 6*). It is more difficult to avoid saturation of the water magnetization in the case of ROESY (*7, 8*). If a water suppression module is appended to a conventional ROESY sequence (e.g., the 1–1 echo ROESY experiment reported by Bax *et al.* (*9*)) the water magnetization will be orthogonal to B_1 during the mixing period and dephased due to RF homogeneity for some steps of the phase cycle required for phase-sensitive detection in F1. Radiation damping during t_1 will also generate a component of the water magnetization orthogonal to B_1 (*10*). The previous schemes for flip-back TOCSY (*5, 6*) are not directly applicable to ROESY experiments, since the latter requires a longer (spin-lock) mixing time and that the magnetization of interest be transverse during the mixing delay. We report here a ROESY pulse sequence (Fig. 1A) in which the water is efficiently returned to equilibrium prior to acquisition by use of a combination of gradient pulses, intentional radiation damping, and selective RF pulses. We have avoided the use of selective excitation at the solvent frequency prior to the mixing period, in order to preserve information at this frequency along F1, and we have introduced minimal additional delays to avoid sensitivity losses due to relaxation. Because radiation damping effects are controlled, efficient recovery of water *z*-magnetization is obtained for the entire phase cycle and over the entire range of t_1 evolution delays.

The flip-back ROESY sequence is shown in Fig. 1A. During the evolution period a bipolar pair of weak gradient pulses G_1 is used to reversibly quench radiation damping (*10*), and thus minimize line broadening at the water frequency along F1 (*11*). Following the evolution period is a *z*-filter containing delay τ_z for intentional radiation damping and a homospoil gradient pulse G_2. The water magnetization is purely along the *z*-axis by the end of the *z*-filter; i.e., the phase modulation imposed by TPPI is selectively removed from the water magnetization. The excitation pulse phase ϕ_1 has been shifted by 45° to minimize the length of τ_z (*12–15*). The optimum value for τ_z depends upon the spectrometer frequency and probe tuning, but can be easily calculated by use of Eqs. [1] and [3] (see below). Following the *z*-filter the water magnetization is returned to the transverse plane and is immediately spin-locked, again suppressing radiation damping (*16*). A CW spin-lock pulse is used during the mixing period, flanked by two hard 90° pulses phased along *y* to compensate for resonance-offset effects on the transverse NOE intensities (*17*). The use of the spin lock to ''store'' the water magnetization during mixing is preferable for ROESY to the approach used in (*6*) for TOCSY, where transverse water magnetization is dephased by a gradient pulse prior to isotropic mixing and rephased afterward. The latter approach would result in significant loss of water magnetization during typical ROESY mixing times due to molecular diffusion (*18, 19*). Following the mixing period is a second *z*-filter containing a homospoil gradient pulse G_3 and a 90° selective flip-back RF pulse,

[1] Present address: Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

[2] To whom correspondence should be addressed.

1090-7807/97 $25.00

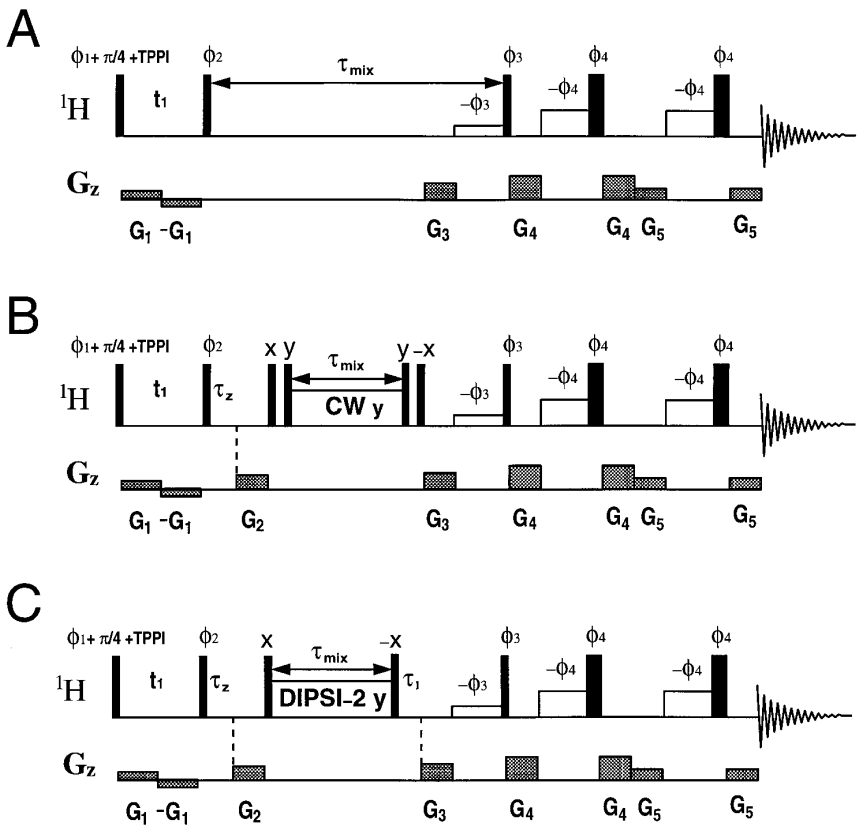

FIG. 1. Pulse sequences used in this work. (A) ROESY with *z*-filters, water flip back, and water suppression via excitation sculpting. (B) NOESY with water flip back and excitation sculpting. (C) TOCSY with *z*-filters, water flip back, and excitation sculpting. The transmitter is on-resonance at the water frequency. Smaller open boxes (with RF phase $-\phi_3$) denote selective rectangular 90° pulses. Larger open boxes (with RF phase $-\phi_4$) denote selective rectangular 180° pulses. The same phase cycle is used for all three sequences: $\phi_1 = 4(-x, x), 4(y, -y)$; $\phi_2 = 8x, 8y$; $\phi_3 = 2x, 2(-x), 2y, 2(-y)$; $\phi_4 = x, 2(-x), x, y, 2(-y), y$; $\phi_R = x, 2(-x), x, y, 2(-y), y, -x, 2x, -x, -y, 2y, -y$. Gradient strengths (in G cm^{-1}): $G_1 = 0.25$, $G_2 = 12$, $G_3 = 9$, $G_4 = 7.2$, and $G_5 = 6$. All gradient pulses are rectangular and 600 μs in duration except G_1 pulses which are of duration $t_1/2$; i.e., their duration is incremented during the 2D experiment. Each gradient pulse, except G_1, is followed by a 100-μs recovery delay.

which maintains the water magnetization along the *z*-axis after the read pulse. Finally, an excitation sculpting module (*20, 21*), containing simple selective inversion elements (soft 180°–hard 180°), dephases any remaining transverse magnetization at the water frequency.

WATERGATE (*22, 23*) may also be used in this sequence with good results. However, the excitation sculpting scheme offers certain advantages. The selective 180° pulses may be calibrated in a simple one-pulse experiment and used without further adjustment. The phase difference between the high and low RF power levels need not be compensated. The total length of the excitation sculpting module is similar to that of WATERGATE (6 to 8 ms), and transverse relaxation losses are therefore similar.

The key to the flip-back ROESY experiment is the use of intentional radiation damping during τ_z. The time required for efficient radiation damping is minimized by phase shifting the excitation pulse by 45°, as is done in some implementations of the flip-back NOESY experiment (*12, 14*), so that following a second pulse the water is never tilted more than 135° with respect to the *z*-axis. The phase shift causes no baseline or phase distortion and no loss of sensitivity and merely adds 45° to the zero-order phase correction in F1. The minimum time τ_z required for recovery of water *z*-magnetization from a flip angle θ is a function of the radiation damping time constant τ_r, which in turn is dependent on the ^{1}H Larmor frequency and probehead impedance matching (*16, 24*). Since the relaxation of solvent water magnetization is dominated by radiation damping, other spin relaxation mechanisms may be neglected and τ_r may be measured directly from the water linewidth (*10*):

$$\tau_r = \frac{0.8384}{\pi \Delta \nu_{1/2}}. \qquad [1]$$

The recovery time τ_z of solvent *z*-magnetization as a function of θ is given by (*25, 26*)

$$\tau_z = \tau_r \left[\tanh^{-1} \frac{M_z}{M_0} + \ln\left(\tan \frac{\theta}{2} \right) \right]. \qquad [2]$$

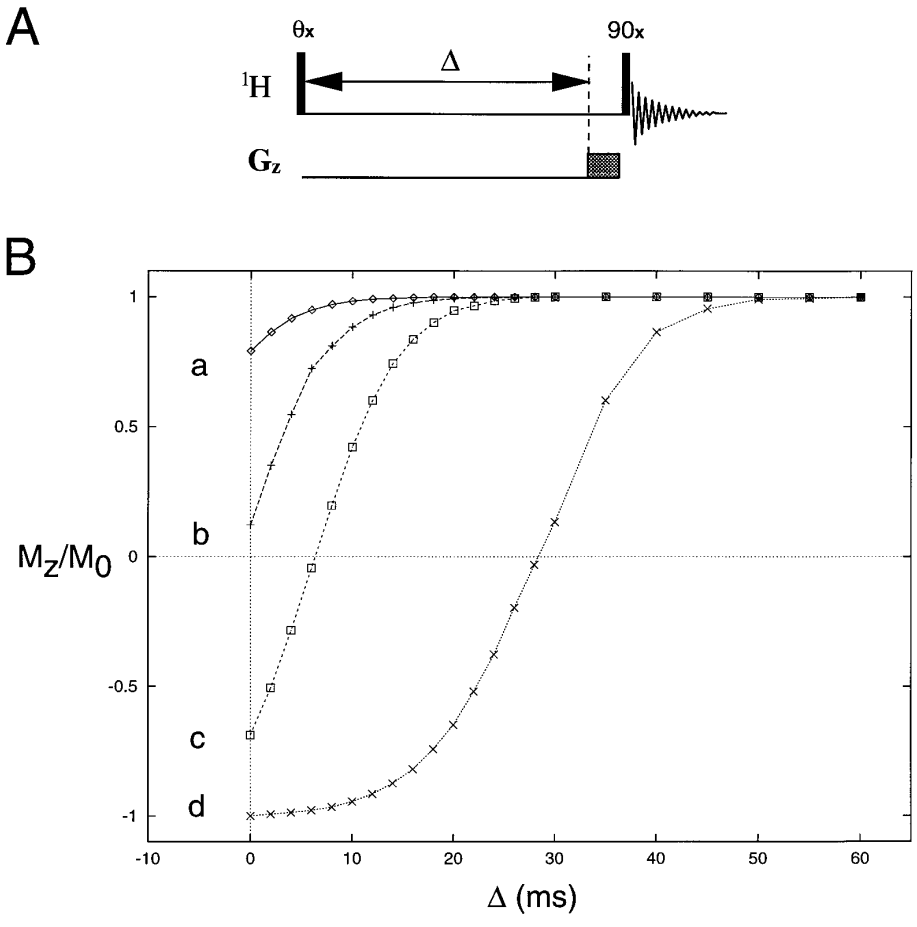

FIG. 2. Recovery of water *z*-magnetization via radiation damping vs flip angle θ. (A) The pulse sequence used. (B) Recovery curves: (a) $\theta = 45°$, (b) $\theta = 90°$, (c) $\theta = 135°$, (d) $\theta = 180°$. M_z/M_0 values were obtained by integrating the water peak. In the $\theta = 135°$ case recovery is complete by 25 ms, in agreement with the value of τ_z calculated by use of Eqs. [1] and [3].

In our experience, good performance of the sequences described in Fig. 1 results if 95% recovery of water magnetization is obtained during delay τ_z. The required delay for $M_z/M_0 = 95\%$ and $\theta = 135°$ is given by

$$\tau_z(95\%) = 2.71\tau_r. \qquad [3]$$

At 500 MHZ a typical water linewidth is 30 Hz, giving $\tau_r = 8.9$ ms and $\tau_z(95\%) = 24$ ms. This calculated value has been verified by experimental calibration of τ_z (see Fig. 2). Longitudinal cross-relaxation during τ_z may in principle cause distortion of ROE intensities. However, in the case of medium-sized biomolecules such as peptides, where ROESY mixing times are typically on the order of 250 ms for maximum buildup of transverse NOE intensity, a 24-ms longitudinal period will not interfere significantly. At higher field strength radiation damping is significantly faster and even shorter delays can be used (e.g., at 750 MHz, τ_z = ca. 11 ms).

In Figs. 1B and 1C the corresponding NOESY and TOCSY sequences are shown, which are identical to the ROESY sequence except for the mixing scheme. The three sequences employ identical RF and gradient programs and a standard NOESY phase cycle. Therefore, minimal parameter adjustment is required once one of the experiments has been set up.

The NOESY sequence is identical to the original flip-back NOESY reported by Lippens *et al.* (*3*) except for the use of gradients during evolution and excitation sculpting for water suppression. In addition, because the excitation pulse is phase shifted, flip-back NOESY spectra can be acquired with mixing times as short as 30 ms at 500 MHz (*27*). Such NOESY data are useful for generating NOE buildup curves, and for distinguishing between direct NOE contacts and spin-diffusion-mediated contacts.

The TOCSY variant (Fig. 1C) requires an isotropic mixing sequence that can be phased entirely along $\pm y$ in order to spin-lock the water magnetization. If a mixing scheme without intrinsic compensation for cross-relaxation effects is used (e.g., WALTZ-16 (*28*), DIPSI-2 (*29*)), then longitudinal cross-relaxation during τ_z and uncompensated transverse cross-relaxation during τ_{mix}

will effectively cancel, as occurs in some schemes for ''clean'' TOCSY experiments (*30–32*). Delay τ_1 (Fig. 1C) may be adjusted to optimize the cancellation, and thereby remove cross-relaxation artifacts. In the slow-motional regime ($\omega_0\tau_c \gg 1$), usually applicable to proteins, the transverse (σ^r) and longitudinal (σ^n) cross-relaxation rates are related by (*33*)

$$\sigma^r = -2\sigma^n. \quad [4]$$

Neglecting off-resonance effects, the effective transverse relaxation rate during DIPSI-2 mixing is $(0.25)\sigma^n$ (*34*). Therefore the optimal value of τ_1 for slow-motional regime systems is given by

$$\tau_1 = \frac{\tau_{mix}}{2} - \tau_z. \quad [5]$$

At very high fields ($\nu_0 \geq 750$ MHz), where τ_z is small, it is preferable to omit τ_1 and use an intrinsically compensated isotropic mixing scheme such as TOWNY-16 (*35*).

Experiments were performed at 500 MHz on a Bruker AMX500 spectrometer using a 5-mm $^1H-^{13}C-^{15}N$ probehead equipped with actively shielded *z*-gradients. Figure 3A shows a ROESY spectrum obtained by use of the flip-back ROESY sequence (Fig. 1A) of a 2 m*M* 12-residue peptide in 90% H_2O at pH 6.9. Figure 3B shows the corresponding flip-back TOCSY (Fig. 1C) spectrum of the same sample. The water suppression is excellent; the residual solvent signal has only very weak dispersive tails. Cross peaks are well resolved at the solvent frequency.

The intentional use of radiation damping during τ_z is equivalent to a narrowband selective RF pulse at water frequency (*36*) and might be expected to bleach or distort cross peaks near this frequency. In fact, the ROESY and TOCSY spectra obtained by use of sequences 1A and 1C are very clean at water frequency in F1 (Figs. 3A and 3B). The RF bandwidth of radiation damping is very narrow, so that only signals on-resonance with water will be affected. The average effective RF field during radiation damping $\langle\nu_1^{rd}\rangle$ is given by (*16, 26*)

$$\langle\nu_1^{rd}\rangle = \frac{1}{2\pi\tau_r\tau_z}\int_0^{\tau_z} \operatorname{sech}\left\{\frac{t}{\tau_r} - \ln\left[\tan^{-1}\left(\frac{\theta}{2}\right)\right]\right\}dt. \quad [6]$$

Under the experimental conditions of Fig. 3 ($\tau_r = 8.9$ ms, $\tau_z = 25$ ms, maximum $\theta = 135°$) Eq. [6] gives $\langle\nu_1^{rd}\rangle = 13.1$ Hz, i.e., on the order of the proton linewidth for biomolecules. Furthermore, radiation damping during τ_z removes the TPPI-induced phase modulation in t_1 at the water frequency, but does not attenuate the magnetization amplitude (*24*). The sign discrimination of frequency along F1 will be lost for these cross peaks, but this is of no consequence at zero offset.

In conclusion, we have demonstrated a variant of the ROESY experiment with minimal saturation of the water magnetization and yet very efficient suppression of the water signal during acquisition. The use of intentional radiation damping prior to mixing is a feasible means of achieving this result, especially in high-field NMR. The flip-back ROESY sequence and the corresponding NOESY and TOCSY sequences were designed so that most experimental parameters are not sample or mixing-mode dependent, and in practice the sequences are convenient to use on a routine basis.

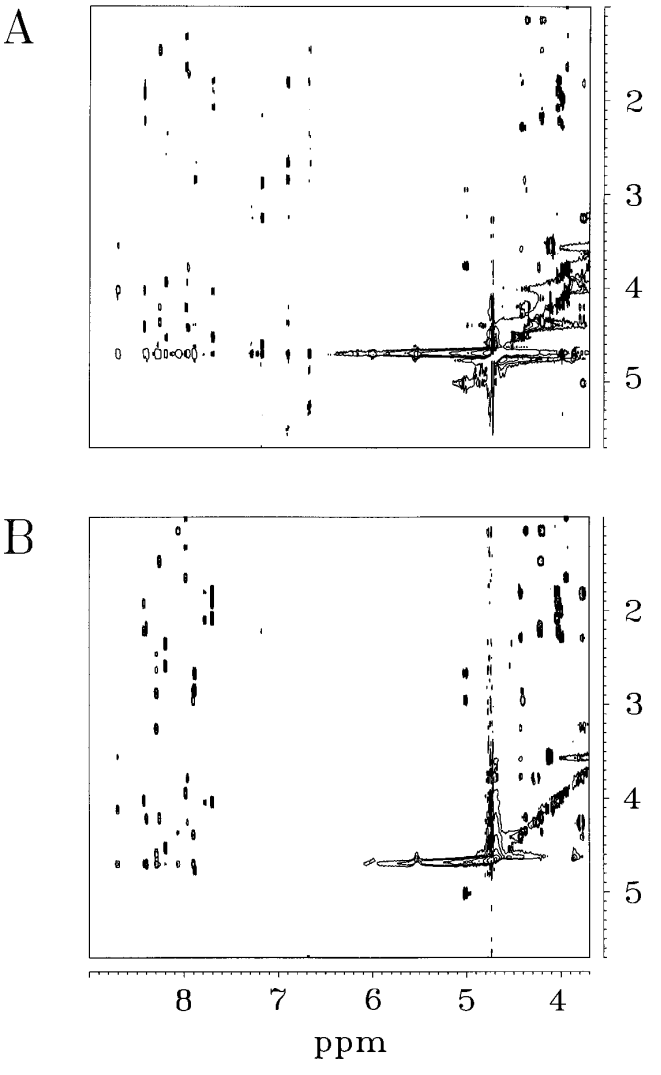

FIG. 3. NMR spectra of a 12-residue peptide in 2 m*M* aqueous solution (90% H_2O/10% D_2O, $T = 25°C$, pH 6.9) showing fingerprint and solvent regions. (A) Flip-back ROESY. (B) Flip-back TOCSY. In (A) positive and negative intensities are shown; positive intensity is depicted by a single contour. In (B) only positive intensity is shown. Selective pulse power level: 90 Hz. For ROESY data: CW spin-lock power = 5 kHz, $\tau_{mix} = 250$ ms. For TOCSY data: DIPSI-2 spin-lock power = 25 kHz, $\tau_{mix} = 60$ ms. For both spectra: 64 scans/FID, 2048 complex data points in t_2, 250 increments in t_1. Spectra were zero-filled to yield 2048 × 1024 real points after processing. Window function: 60°-shifted sine bell. No postacquisition solvent suppression was used.

ACKNOWLEDGMENT

We thank Dr. Ping Xu for providing the peptide sample, and for stimulating discussions. This paper is NRCC Publication No. 39981.

REFERENCES

1. F. J. Moy, H. A. Scheraga, S. L. Patt, and G. T. Montelione, *J. Magn. Reson.* **98,** 451–457 (1992).
2. S. Grzesiek and A. Bax, *J. Am. Chem. Soc.* **115,** 12593–12594 (1993).
3. G. Lippens, C. Dhalluin, and J.-M. Wieruszeski, *J. Biomol. NMR* **5,** 327–331 (1995).
4. D. B. Fulton, R. Hrabal, and F. Ni, 36th Experimental NMR Conference, Boston, MA (1995).
5. C. Dhalluin, J.-M. Wieruszeski, and G. Lippens, *J. Magn. Reson. B* **111,** 168–170 (1996).
6. D. B. Fulton, R. Hrabal, and F. Ni, *J. Biomol. NMR* **8,** 213–218 (1996).
7. A. A. Bothner-By, R. L. Stephens, J. Lee, and C. D. Warren, *J. Am. Chem. Soc.* **106,** 811–813 (1984).
8. A. Bax and D. G. Davis, *J. Magn. Reson.* **63,** 207–213 (1985).
9. A. Bax, V. Sklenar, G. M. Clore, and A. M. Gronenborn, *J. Am. Chem. Soc.* **109,** 6511–6513 (1987).
10. V. Sklenar, *J. Magn. Reson. A* **114,** 132–135 (1995).
11. G. Otting, *J. Magn. Reson. B* **103,** 288–291 (1994).
12. O. Zhang, L. E. Kay, J. P. Olivier, and J. D. Forman-Kay, *J. Biomol. NMR* **4,** 845–858 (1994).
13. J. Stonehouse, R. T. Clowes, G. L. Shaw, J. Keeler, and E. D. Laue, *J. Biomol. NMR* **5,** 226–232 (1995).
14. S. Talluri and G. Wagner, *J. Magn. Reson. B* **112,** 200–205 (1996).
15. D. L. Mattiello, W. S. Warren, L. Mueller, and B. T. Farmer II, *J. Am. Chem. Soc.* **118,** 3253–3261 (1996).
16. W. S. Warren, S. L. Hammes, and J. L. Bates, *J. Chem. Phys.* **91,** 5895–5904 (1989).
17. C. Griesinger and R. R. Ernst, *J. Magn. Reson.* **75,** 261–271 (1987).
18. E. O. Stejskal and J. E. Tanner, *J. Chem. Phys.* **42,** 288–292 (1965).
19. J. Keeler, R. T. Clowes, A. L. Davis, and E. D. Laue, *Methods Enzymol.* **239,** 145–207 (1994).
20. T.-L. Hwang and A. J. Shaka, *J. Magn. Reson. A* **112,** 275–279 (1995).
21. D. Callihan, J. West, S. Kumar, B. I. Schweitzer, and T. M. Logan, *J. Magn. Reson. B* **112,** 82–85 (1996).
22. M. Piotto, V. Saudek, and V. Sklenar, *J. Biomol. NMR* **2,** 661–665 (1992).
23. V. Sklenar, M. Piotto, R. Leppik, and V. Saudek, *J. Magn. Reson. A* **102,** 241–245 (1993).
24. N. Bloembergen and R. V. Pound, *Phys. Rev.* **95,** 8–12 (1954).
25. S. Bloom, *J. Appl. Phys.* **28,** 800–805 (1957).
26. X. Mao and C. Ye, *J. Chem. Phys.* **99,** 7455–7462 (1993).
27. D. B. Fulton and F. Ni, unpublished results.
28. A. J. Shaka, J. Keeler, T. A. Frenkiel, and R. Freeman, *J. Magn. Reson.* **52,** 335–338 (1983).
29. A. J. Shaka, C. J. Lee, and A. Pines, *J. Magn. Reson.* **77,** 274–293 (1988).
30. C. Griesinger, G. Otting, K. Wütrich, and R. R. Ernst, *J. Am. Chem. Soc.* **110,** 7870–7872 (1988).
31. D. W. Bearden, S. Macura, and L. R. Brown, *J. Magn. Reson.* **80,** 534–538 (1988).
32. D. Marion, P. C. Driscoll, L. E. Kay, P. T. Wingfield, A. Bax, A. M. Gronenborn, and G. M. Clore, *Biochemistry* **28,** 6150–6156 (1989).
33. J. Briand and R. R. Ernst, *Chem. Phys. Lett.* **185,** 276–285 (1991).
34. C. Griesinger and R. R. Ernst, *Chem. Phys. Lett.* **152,** 239–247 (1988).
35. M. Kadkhodael, T.-L. Hwang, J. Tang, and A. J. Shaka, *J. Magn. Reson. A* **105,** 104–107 (1993).
36. G. Otting and E. Liepinsh, *J. Biomol. NMR* **5,** 420–426 (1995).