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Multiple-Pulse Mixing Sequences That Selectively Enhance Chemical Exchange or Cross-Relaxation Peaks in High-Resolution NMR Spectra

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Rotating-frame NMR experiments which either emphasize or suppress cross relaxation, and which simultaneously suppress TOCSY, COSY, and zero-quantum peaks in NMR spectra, are presented and analyzed. The new experiments rely on mixing sequences which follow naturally from the *transverse*-ROESY (Tr-ROESY) sequence of Hwang and Shaka, and which are applicable to larger molecules in solution (spin diffusion limit). In the first variant a modified Tr-ROESY sequence, called multiple-pulse ROESY (MP-ROESY), is used to enhance cross-relaxation peak intensity compared to Tr-ROESY; in the second, called phase-modulated CLEAN chemical exchange (CLEANEX-PM), cross-relaxation peaks are greatly attenuated. The two methods are thus complementary: MP-ROESY is used to observe Overhauser peaks, and CLEANEX-PM is used to eliminate them, permitting clear observation of chemical exchange peaks alone. The new techniques are examined by theory and experiment. Practical guidelines that will result in high-quality spectra are given, including the judicious use of continuous weak static magnetic field gradients. © 1998 Academic Press

Key Words: **chemical exchange; CLEANEX-PM; cross relaxation; MP-ROESY; magnetic field gradient; zero quantum dephasing.**

INTRODUCTION

In the phase-sensitive 2D ROESY (*1, 2*) experiment, both coherent and incoherent magnetization transfer may occur (*1–4*). Coherent magnetization transfer between *J*-coupled spins is undesirable and can be classified broadly as either "TOCSY-type" or "COSY-type" (*2, 5–7*). The two types can be distinguished by their different cross peak patterns: TOCSY-type cross peaks are mostly in phase with the diagonal peaks and have nonzero integral over the 2D multiplet, while the integral of antiphase COSY-type cross peak multiplets vanishes. Both mechanisms can give unwanted cross peaks in the ROESY experiment. Of the two, however, TOCSY is the more confusing because relayed ROEs, involving a cross relaxation step followed or preceded by a TOCSY step, are of the same polarity as true ROEs and are misleading (*8*). Incoherent magnetization transfer pathways include both cross relaxation and chemical exchange (*9*). The algebraic sign of chemical exchange cross peaks is opposite that of the ROE cross peaks, making it possible to identify cross peaks caused by the different mechanisms (*1, 3*) provided that they are resolved. Sequential magnetization transfer through chemical exchange and cross relaxation can once again lead to additional unwanted peaks. When the spectral features are not completely resolved, the unwanted peaks can greatly complicate the spectral analysis by causing partial cancelation of the desired signals.

The embarrassing abundance of magnetization transfer that can occur in ROESY makes it highly desirable to design experiments to observe *only one effect at a time*. Measuring the cross-relaxation rates while suppressing the TOCSY transfer in ROESY has been an active area of research (*2, 10–17*). We have proposed the *transverse*-ROESY (Tr-ROESY[3]) experiment (*13, 15*), in which original spin locking field SL_y during the mixing time is replaced with a windowless sequence of phase alternating 180° pulses $[180°(x)\ 180°(-x)]_n$, and shown its effectiveness with regard to TOCSY suppression: Tr-ROESY suppresses most TOCSY transfer and averages the transverse and longitudinal cross-relaxation rates. For macromolecules, the transverse and longitudinal cross-relaxation rates have opposite signs, and so the buildup of cross peak intensity is slower in Tr-ROESY than in conventional ROESY. For smaller molecules, however, the buildup curves of Tr-ROESY and of ROESY are roughly the same, as the transverse and longitudinal cross-relaxation rates are of the same sign. In this paper we outline a modified version of Tr-ROESY that gives faster ROE buildup at the cost of some TOCSY suppression, an improvement that may be of interest in the spectroscopy of larger molecules at very high magnetic fields. We call this sequence multiple-pulse rotating-frame Overhauser effect spectroscopy (MP-ROESY).

Another possibility in the rotating-frame experiments is a "pure" chemical exchange spectrum. Although cross peaks caused by different incoherent magnetization transfer mecha-

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[3] In order not to confuse with 3QF T-ROESY ("T" refers to *T*ilted frame) (*43*), we have changed the abbreviation of transverse-ROESY to Tr-ROESY.

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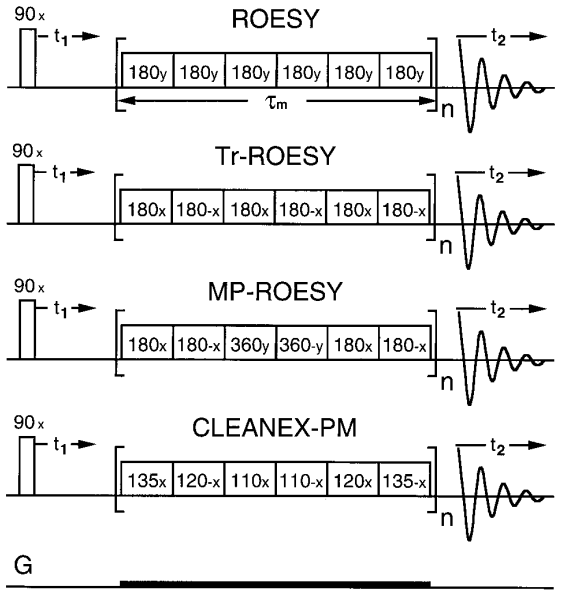

FIG. 1. Pulse sequence timing diagrams for different rotating-frame 2D experiments. In the mixing period, a multiple-pulse sequence is used to control and manipulate cross relaxation. The absorption-mode 2D spectrum is achieved by employing a small gradient throughout the mixing period.

nisms can be distinguished in ROESY, eliminating both scalar coupling and cross-relaxation transfer clarifies the chemical exchange spectrum. We have designed a robust mixing sequence to accomplish this aim, which we call phase-modulated clean chemical exchange spectroscopy (CLEANEX-PM) (*18*). It is an improvement of a previously described amplitude-modulated mixing sequence devised for the same purpose and which is referred to as CLEANEX-AM (*19*).

We will refer to a number of different pulse sequences, with timing diagrams laid out in Fig. 1. The sequence for MP-ROESY is designed to increase the observed cross-relaxation rate for macromolecules, in which the performance is a compromise between conventional ROESY and Tr-ROESY. The sequence for CLEANEX-PM is intended to inhibit *both* the scalar coupling and cross-relaxation magnetization transfers in exchange spectroscopy, resulting in an unambiguous chemical exchange spectrum. The particular CLEANEX-PM implementation in Fig. 1 is appropriate for the spin diffusion limit.

THEORY

The Scaling Factor and Effective Cross Relaxation Rate

When a pair of *J*-coupled proton spins are spin locked along the *y*-axis of the rotating frame by a periodic pulse sequence, the possibility of TOCSY magnetization transfer can be calculated from a simple geometrical picture using a fictitious 3D vector model which has been discussed in detail (*15, 20*). The actual chemical shift difference between the two spins is scaled by some factor λ between zero and unity under the action of the sequence and serves the role of a resonance offset term in this model. The scalar coupling, J, provides a "transverse field." Difference magnetization ($I_{1y} - I_{2y}$) evolves under the influence of the vector sum of these two "fields" according to a simple torque equation. If the effective chemical shift difference is small, either because the true chemical shift difference is small or because the scaling factor λ is small, then J has the ability to invert the ($I_{1y} - I_{2y}$) state, much like a soft 180° pulse can invert spins near resonance. The inversion of the ($I_{1y} - I_{2y}$) state is nothing other than TOCSY (*5*). To prevent TOCSY transfer, the mixing sequence should thus have a nonzero scaling factor over the bandwidth of interest: the closer λ is to unity, the smaller the percentage of unwanted TOCSY transfer. The scaling factor λ can be calculated for any periodic multiple-pulse sequence by applying the sequence to an isolated spin and using the formula (*21*)

$$\lambda = \frac{1}{t_s}\frac{\partial \beta}{\partial \omega}, \qquad [1]$$

where β is the net rotation angle of the sequence, t_s is its duration, and ω is the resonance offset; λ measures how much the chemical shift range is locally compressed under the action of the multiple-pulse sequence. Note that only the performance of the sequence at the end of one entire period is important; it is not necessary to follow the trajectories in a blow-by-blow fashion. Decoupling sequences, for example, strive to achieve $\lambda = 0$ and are thus of very little use for ROESY or exchange spectra. If, on the other hand, the difference of the effective resonance offsets between two coupled spins is large compared to J, the TOCSY transfer can be effectively suppressed.

The suppression of cross-relaxation peaks is a different kind of problem. Basically, the longitudinal and transverse relaxation rates operate *throughout* the entire pulse sequence, making it necessary to follow the spin trajectory in detail. The theory is well understood for the simple case of a pair of spins held rigidly at a fixed distance and undergoing isotropic reorientation with single correlation time. The longitudinal and transverse cross-relaxation rates are (*1, 22, 23*)

$$(\sigma_{ln})_{ij} = \frac{\gamma^4\hbar^2}{10r_{ij}^6}\left(\frac{6}{1+4\omega_0^2\tau_c^2} - 1\right)\tau_c \qquad [2]$$

$$(\sigma_{tr})_{ij} = \frac{\gamma^4\hbar^2}{10r_{ij}^6}\left(\frac{3}{1+\omega_0^2\tau_c^2} + 2\right)\tau_c, \qquad [3]$$

where $\omega_0/2\pi$ is the spectrometer frequency, τ_c is the correlation time of isotropic reorientation, and r_{ij} is the fixed distance between the two spins. In a high-field NMR spectrometer, biomolecules with long τ_c can reach the "spin diffusion" limit, $\omega_0\tau_c \gg 1$. This leads to $\sigma_{tr} = -2\sigma_{ln}$. If the molecular motion and magnetic field strength conspire to put the molecule in the intermediate regime, $\omega_0\tau_c \sim \sqrt{5}/2$, then σ_{ln} vanishes and some

sort of ROESY-type experiment is necessary. When a multiple-pulse sequence is operating, the spins are rapidly but coherently reoriented along different directions and the observed cross relaxation rate $\bar{\sigma}$ becomes some weighted average of σ_{ln} and σ_{tr}. Griesinger and Ernst developed the invariant trajectory method to calculate the effective cross-relaxation rate for a two-spin system under the influence of a multiple-pulse sequence (*24*). Their formula, applied to a periodic multiple-pulse sequence, is

$$\bar{\sigma} = \frac{1}{\tau_s}\int_0^{\tau_s} (\sigma_{tr}\{m_{1x}(t)\cdot m_{2x}(t) + m_{1y}(t)\cdot m_{2y}(t)\} + \sigma_{ln} m_{1z}(t)\cdot m_{2z}(t))dt, \quad [4]$$

where m_{1x} is the projection of the first spin on the x-axis, etc., and the integration is carried out over one period. During the mixing period, the projections of these two interacting spins on the xy-plane undergo transverse cross relaxation, and the projections on the z-axis are subject to the longitudinal cross relaxation. Any other relaxation effects are neglected in this calculation. If $\sigma_{tr} = -2\sigma_{ln}$, the longitudinal and transverse cross-relaxation rates can be canceled as long as the spins spend twice as much time on the z-axis as on the xy-plane. This idea has been used to design CLEAN TOCSY and chemical exchange sequences. Previously proposed methods use three approaches to cancel the cross relaxation rates. The first is to insert delays into the pulse sequence at appropriate times, letting the spins spend longer on the z-axis (*25–29*). The second is to apply an amplitude-modulated B_1 field to move the spins at different rates according to the positions of the spins (*19, 30*). The third uses a constant B_1 field in conjunction with phase modulation so that the spins spend less time on the xy-plane compared to the z-axis (*31*).

To assess a given sequence, it is convenient to compute both its scaling factor and its effective cross relaxation rate. It is convenient also to identify the net rotation axis **n** of the sequence, to make sure it is closely aligned with the y-axis. These three items can be obtained from a numerical integration of an ensemble of isolated spins, as a function of resonance offset, under the pulse sequence. It is not necessary to simulate coupled spin systems. Once λ, $\bar{\sigma}$, and **n** are known, a comparison between competitive sequences can be made. The choice may depend somewhat on the molecule under study. For ROESY-type experiments on a small natural product in the intermediate regime, where many spins are coupled and close in chemical shift, a very large scaling factor may be most important. For a polypeptide with good shift dispersion but closer to the spin diffusion limit, a large $\bar{\sigma}$ may be most important. For sequences with similar performance, additional criteria such as ease of implementation, tolerance to pulse miscalibration, etc., can be used to make a final choice.

Phase-Sensitive Spectra

In 2D spectroscopy it is very important to be able to obtain a spectrum with absorption- or near-absorption-mode line shapes in both F_1 and F_2. It is well known that this usually requires two amplitude-modulated data sets in which the sine and cosine modulation have been recorded separately (*32*). As a corollary, it must therefore be possible to phase the very first increment to nearly pure absorption, so that it looks close to a spectrum obtained after a single 90° pulse. Linear frequency phase corrections are undesirable because they can distort the baseline, making contour plots difficult and volume integrals less reliable.

In conventional ROESY, the natural inhomogeneity of the B_1 field over the sample volume leads to relatively rapid dephasing of magnetization that is perpendicular to the spin lock field. If the B_1 field is strong, this dephasing of either the x- or y-component of the magnetization is efficient and can be used naturally to provide the pair of amplitude modulated data sets required for a phase-sensitive spectrum. Unfortunately, a strong B_1 field gives a small scaling factor and can therefore increase the size of TOCSY peaks. Using a weaker off-resonance spin locking field results in less efficient dephasing because the actual range of nutation frequencies experienced by an off-resonance spin is somewhat smaller and the number of revolutions per unit time is less. There is also some loss of sensitivity, as a B_1 field tilted by an angle θ from the y-axis will dephase part of the desired y-magnetization, giving a $\cos^2\theta$ loss. Griesinger and Ernst (*33*) recognized these problems and suggested bracketing the spin lock by a pair of strong 90° pulses, e.g., 90°(y)–SL(y)–90°(y). The first pulse aligns all transverse magnetization in the yz-plane and the second returns all magnetization from the yz-plane to the xy-plane. If the 90° pulses are strong and correctly calibrated, the amplitude loss is converted to a mild linear phase correction in each dimension. In addition, some unwanted coherence transfer pathways arising from the tilted spin lock field are eliminated by the 90° pulses (*33*).

For the sequences we propose here, the 90° pulses are necessary for a different reason. The net rotation axis of the Tr-ROESY mixing sequence $[180°(x)\ 180°(-x)]_n$ is closely aligned along the y-axis, so that tilted field effects do not enter in. However, near resonance the $[180°(x)\ 180°(-x)]_n$ sequence forms rotary echoes (*34*), which results in *both* x- and y-magnetization being retained. Thus, to obtain a proper phase-sensitive Tr-ROESY spectrum, the bracketing 90°(y) pulses are required, and all four combinations $90(\pm y)[180°(x)\ 180°(-x)]_n 90(\pm y)$ should be used. This additional phase cycling can be avoided by making use of weak continuous static field gradients (see below). The same comments apply to the other sequences we will introduce.

Enhancing the Cross-Relaxation Rate: MP-ROESY

Assuming that σ_{tr} and σ_{ln} are of opposite sign, then the longitudinal and transverse cross-relaxation rates will tend to cancel, and $\bar{\sigma}$ will be less than σ_{tr}. For Tr-ROESY, we have shown (*13*) that, reasonably close to the transmitter offset,

$$\bar{\sigma}_{ij} = \frac{(1 + \sin\theta_i \sin\theta_j)(\sigma_{tr})_{ij} + \cos\theta_i \cos\theta_j (\sigma_{ln})_{ij}}{2} \quad [5]$$

$$\lambda = \frac{2}{\pi} \frac{1}{1 + (\Delta\omega/\gamma B_1)^2} \quad [6]$$

and

$$\mathbf{n} \approx (0, 1, 0), \quad [7]$$

where $\theta_i = \tan^{-1}(\Delta\omega_i/\gamma B_1)$ is the tilt angle of the effective field. In the spin diffusion limit $\bar{\sigma}_{ij}/\sigma_{tr} = 0.25$ near resonance, while $\lambda = 2/\pi \approx 0.637$. A simple way to enhance the effective cross relaxation is to insert conventional y-pulses into Tr-ROESY. To avoid perturbing the net axis of the resulting sequence, the additional pulses should approximate a cyclic sequence. These considerations suggest the simple sequence $360°(y)\ 360°(-y)$ as a candidate, giving

$$R_{MP-ROESY} = 180°(x)180°(-x)360°(y) \\ 360°(-y)180°(x)180°(-x). \quad [8]$$

Near resonance, the expected performance of MP-ROESY in terms of $\bar{\sigma}$ and λ can be analyzed by realizing that the $360°(y)\ 360°(-y)$ segment is fairly cyclic and so has a net rotation angle of nearly zero and a scaling factor of zero. As such, both $\bar{\sigma}$ and λ become weighted averages for the individual segments, giving

$$\frac{\bar{\sigma}_{MP}}{\sigma_{tr}} = \frac{0.25 + (2 \times 1.0) + 0.25}{4} = 0.625 \quad [9]$$

$$\lambda_{MP} = \frac{2/\pi + (2 \times 0) + 2/\pi}{4} = \frac{1}{\pi} \approx 0.318, \quad [10]$$

showing that the scaling factor is reduced by a factor of two while the effective cross relaxation has increased by *more* than a factor of two. Unfortunately, this simple calculation does not capture the situation adequately off-resonance, nor does it address the detailed dependence of $\bar{\sigma}$ at moderate offsets where the overshoot of the $360°(y)$ pulses are significant. Figure 2a shows a numerical map of $\bar{\sigma}$ for this sequence as a contour plot depending on the resonance offsets of the cross relaxing pair and compares it with that for Tr-ROESY, Fig. 2b. The offset dependence of MP-ROESY is not perfectly symmetric because the underlying sequence is neither purely symmetric nor antisymmetric in time, but the contours are reasonably well behaved and there is a definite increase in $\bar{\sigma}$ near the center of the range.

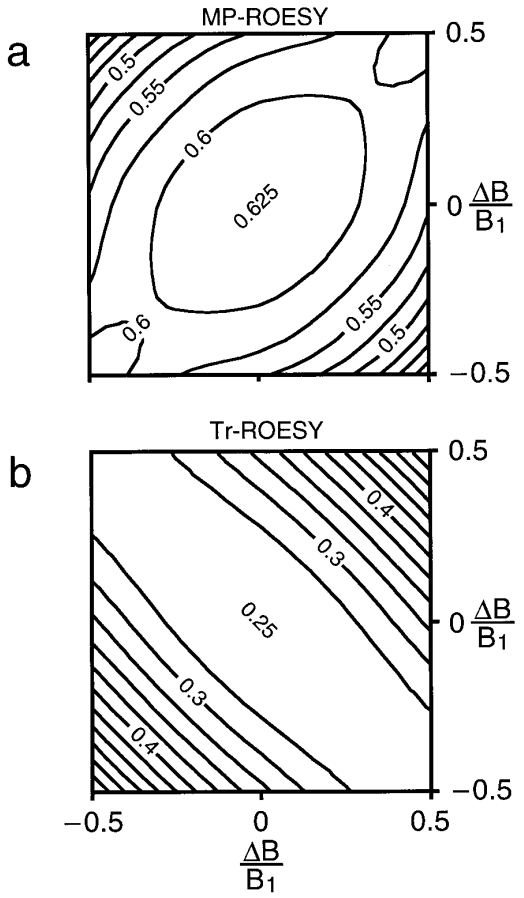

FIG. 2. Contour plots of the effective cross-relaxation rate, $\bar{\sigma}/\sigma_{tr}$, in the spin diffusion limit as a function of the resonance of the two spins. (a), (b) The contours of $180(x)\ 180(-x)\ 360(y)\ 360(-y)\ 180(x)\ 180(-x)$ and $180(x)\ 180(-x)$, respectively.

Suppressing Cross Relaxation: CLEANEX-PM

By arranging that the spins spend more time along the z-axis, $\bar{\sigma}$ can be reduced as long as σ_{tr} and σ_{ln} are of opposite sign. If σ_{ln} is zero ($\omega_0\tau_c \sim \sqrt{5}/2$), then the spins should spend 100% of the time along the z-axis, a conventional NOESY experiment, whereas if $\sigma_{tr} = -2\sigma_{ln}$, then the spins should spend roughly 67% of their time along the z-axis. There is no obvious need for any pulses of phase y in this case, as *less* time along the y-axis is desired, rather than more. The large scaling factor and stable net rotation axis requirements remain the same. A large number of candidate sequences can be located quickly and evaluated, and they differ only in detail. Two promising possibilities for molecules in the spin diffusion limit, using 6 or 12 pulses, are

$$R_{CL-I} = 135°(x)120°(-x)110°(x) \\ 110°(-x)120°(x)135°(-x) \quad [11]$$

and

$$R_{\mathrm{CL-II}} = 160°(x)130°(-x)60°(x)\,40°(-x)90°(x)115°(-x)115°(x)90°(-x)\,40°(x)60°(-x)130°(x)160°(-x). \quad [12]$$

We will refer to the latter sequence as CLEANEX-PM II. The effective cross-relaxation rates for both these sequences are quite small over an extended bandwidth, as shown in Fig. 3. The comparison sequence is the proposed (laboratory frame) mixing sequence of Fejzo *et al.*, —Δ— $90°(x)\ 90°(-x)\ 90°(-x)\ 90°(x)$ —Δ—, where the delay time, Δ, is equal to the 90° pulsewidth (*29*). The CLEANEX-PM sequences null out cross relaxation more efficiently and, as they have large scaling factors, do not give TOCSY peaks either. An important point, however, is that the cancelation presupposes the relationship $\sigma_{\mathrm{tr}} = -2\sigma_{\mathrm{ln}}$. If a small ligand is partially bound to, or in weak association with, a macromolecule, then the effective correlation time may be shorter, leading to detectable ROESY peaks in addition to any exchange peaks; these ROESY peaks will have opposite sign to the exchange peaks. These intermolecular peaks between water and a zinc finger protein have recently been observed (*18*). Quite generally, the CLEANEX-PM sequences can be used to eliminate Overhauser peaks from a large macromolecule and thereby focus on some other weakly interacting ligand that might be present in solution.

Weak Continuous Magnetic Field Gradients During Mixing

As pointed out above, Tr-ROESY and its relatives form rotary echoes near resonance, making an absorption-mode phase-sensitive 2D spectrum unlikely unless the bracketing $90°(\pm y)$ pulses are used. Another way to eliminate x-magnetization is to employ, throughout nearly the entire mixing time, a fairly weak continuous magnetic field gradient. Consider the Tr-ROESY sequence as an example. The net rotation operator over one period of the $180°(x)\ 180°(-x)$ sequence has been shown to be

$$R = \exp\left(-4iI_y\tan^{-1}\left(\frac{\Delta\omega}{\gamma B_1}\right)\right) \quad [13]$$

to first order in $\Delta\omega$. Because the net rotation angle is a nearly linear function of frequency in these sequences, a weak magnetic field gradient sufficient to broaden the lines by ~1 kHz leads to a dispersion in net rotation angles, by about 10° per repetition using a 5-kHz RF field. After several hundred repetitions, as would be typical with a mixing time of 100 ms, the x-component of magnetization is dephased. In theory this gradient should be turned on and off adiabatically, but in practice it is possible simply to turn the gradient on after a few repetitions of the multiple-pulse sequence and turn it off a few repetitions before the conclusion of the mixing time. The net axis for these sequences remains sufficiently aligned along the y-axis over a large range of offsets so that very little in-phase magnetization is lost. Using this method, the $90°(\pm y)$ pulses bracketing the mixing time can be eliminated.

Another advantage of the weak gradient emerges when the

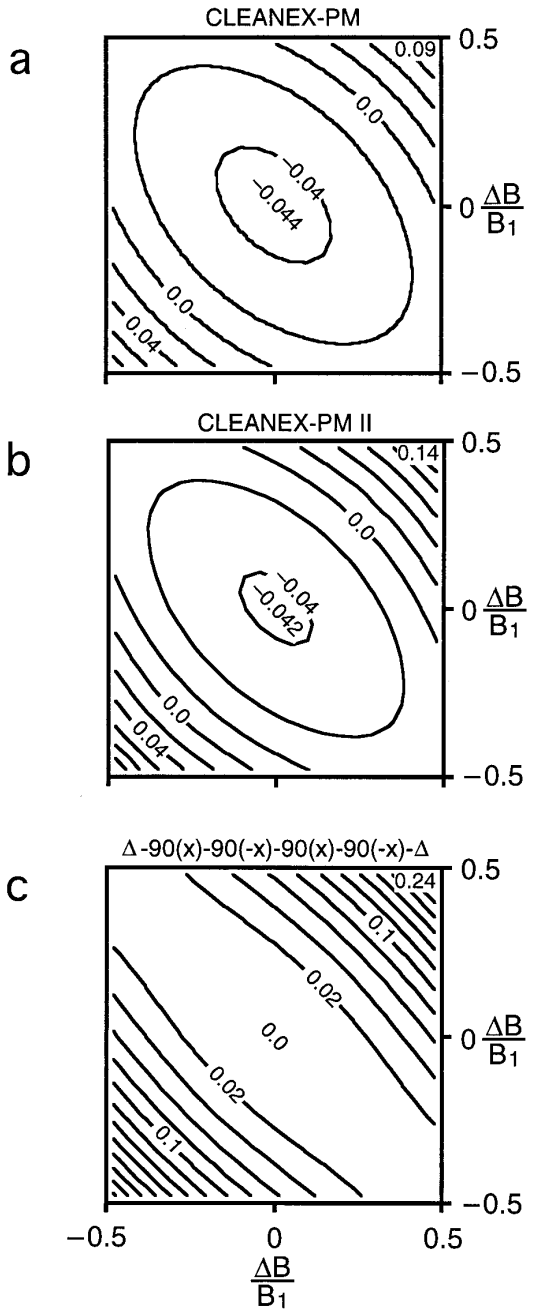

FIG. 3. The effective cross relaxation rate, $\bar{\sigma}/\sigma_{\mathrm{tr}}$, as a function of the resonance offsets of the two interacting spins for the (a) CLEANEX-PM, (b) CLEANEX-PM II, and (c) laboratory-frame sequence. —Δ— $90(x)\ 90(-x)\ 90(-x)\ 90(x)$ —Δ—, where the delay time, Δ, is equal to the 90° pulsewidth. The spin diffusion limit has been assumed. The spacing of contour level is 0.02. The initial magnetization was assumed to be parallel to the effective rotation axis for all the sequences.

effect of zero-quantum (ZQ) coherence on the spectrum is considered. During the evolution time t_1, antiphase spin operators of the form $I_{1x}I_{2z}$, $I_{1y}I_{2z}$, etc., arise between coupled spins. The first term is not removed under the action of a SL(y) sequence and will survive the mixing sequence as a linear combination $c_1I_{1x}I_{2z} + c_2I_{1z}I_{2x}$ leading to weak cross peaks between coupled spins. These COSY-type cross peaks are familiar from the 2D NOESY experiment, where they arise from ZQ coherence (*35, 36*). The ZQ coherence evolves at the chemical shift difference of the two spins involved, so a conventional PFG will *not* remove homonuclear ZQ coherence, in contrast to a recent claim in the literature (*37*). The chemical shift difference between two proton spins is too weak a function of the B_0 field strength for anything but a PFG that is a fair fraction of B_0 *itself* to have any appreciable effect. Such a PFG, in the 10,000 G/cm range, is currently not available for high-resolution systems. However, as the ZQ coherence evolves at the chemical shift difference of the two spins involved, it responds to a scaling of the chemical shift difference under the action of the multiple-pulse sequence. If the scaling factor λ is not completely offset-independent, then, during a weak PFG, the ZQ frequencies become inhomogenous to the extent that λ differs as a function of the actual center offset frequency between the two spins. This dephasing is still pretty slow, but if the PFG is left on throughout the majority of the mixing time, the unwanted cross peaks from ZQ coherence are effectively removed under typical conditions for all weakly coupled spin pairs. This technique is essentially an adaptation of the purging schemes proposed by Davis *et al.* (*36*) and Mitschang *et al.* (*38*) for removing ZQ coherence in NOESY and TOCSY spectra. The economy here is that the purging sequence *is* the mixing sequence.

The last advantage of a long weak PFG arises when carrying out these experiments in aqueous solution. The large H_2O peak, usually placed at the transmitter offset, will have plenty of time for radiation damping to exert its influence during the mixing time. The effect will depend partly on whether the water magnetization trajectory is mostly inverted or mostly at equilibrium during the multiple-pulse sequence. The radiation damping can cause differential pumping of the baseline around the water resonance as a function of t_1, leading to large baseline distortions in the spectrum. A weak PFG that broadens the H_2O resonance to ca. 1 kHz completely stops the radiation damping and leads to high-quality spectra (*18*). Related use of weak PFGs to narrow the H_2O resonance in the F_1 dimension of 2D spectra by extinguishing radiation damping has been proposed previously (*39*).

EXPERIMENTAL

Attenuation of ZQ Peaks Using a Weak Gradient

We tested the ZQ dephasing effect by using the gramicidin-S dissolved in DMSO-d_6 at 25°C on a 500-MHz Varian Unity-Plus spectrometer. The target Phe NH was selectively excited by the excitation sculpting scheme (*40*). Stott *et al.* (*41*) have shown that even a slightly imperfect selective excitation can produce unwanted magnetization, which may lead to artifacts in the final spectrum. Figure 4 shows the dephasing effect of the unwanted magnetization by using either a weak magnetic field gradient or the natural inhomogeneity of the B_1 field. Setting the spin-lock transmitter offset to the Phe NH position, ZQ coherence that was not dephased produced the COSY-type peak at Phe $C_\alpha H$ under MP-ROESY (Fig. 4a). By adding a small gradient, 0.1 G/cm, throughout the mixing period to the same experiment as in Fig. 4a, the COSY-type peaks were dephased, revealing the buildup of Phe $C_\alpha H$ peak (Fig. 4b). When the spin-lock transmitter offset was placed on the resonance of Leu $C_\alpha H$ during the mixing period, the COSY-type peaks were also dephased due to the inhomogeneity of the B_1 field (*38*) (Fig. 4c). The relative intensity of the antiphase COSY-type peak depends on the line shape, the mixing time, the RF field distribution produced by the probe, and the mixing

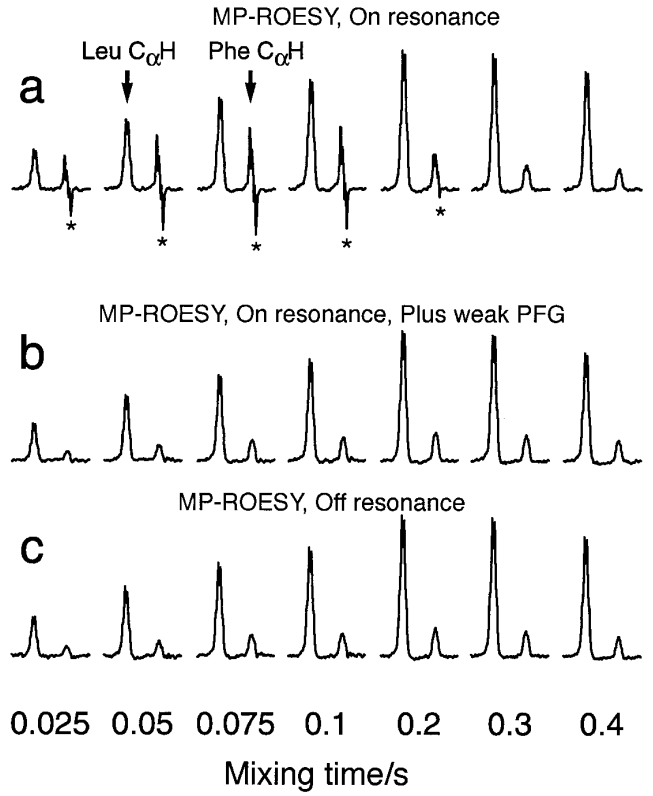

FIG. 4. ZQ dephasing by using a weak magnetic field gradient and the inhomogeneity of the B_1 field for MP-ROESY experiments. After selective excitation of the Phe NH resonance, Leu $C_\alpha H$ and Phe $C_\alpha H$ were observed as a function of mixing time, where Leu $C_\alpha H$ peaks showed a ROE buildup. The spin-lock RF field strength was 5.1 kHz. (a) ZQ peaks at Phe $C_\alpha H$ when the spin-lock transmitter offset was placed at Phe NH. (b) Same as (a) with a small magnetic field gradient on during mixing, leading to ZQ dephasing and revealing the buildup of Phe $C_\alpha H$. (c) ZQ dephasing due to the inhomogeneity of the B_1 field when the spin-lock transmitter offset was placed at Phe $C_\alpha H$.

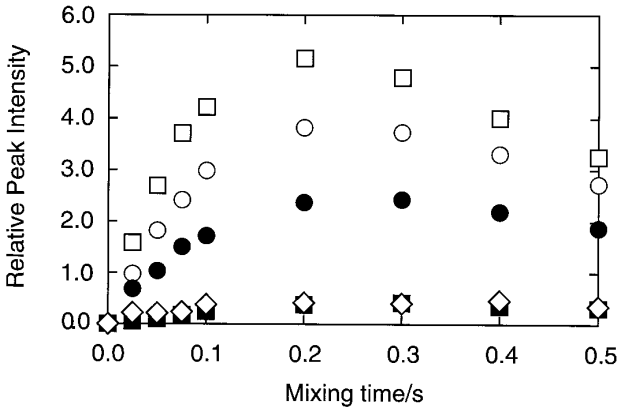

FIG. 5. Plots of the measured ROE buildup of the Leu $C_\alpha H$ resonance in the 1D difference spectrum after selective excitation of the Phe NH resonance. During the spin-lock period, the transmitter offset was placed at Leu $C_\alpha H$. (□) ROESY, (○) MP-ROESY, (●) Tr-ROESY, (■) CLEANEX-PM, (◇) CLEANEX-PM II.

sequence, as well as the relaxation behavior of the molecule itself. For instance, when using CLEANEX-PM in the experiment performed for Fig. 4a, the COSY-type peaks have higher intensity and persist longer because the scaling factors of the sequence do not change as rapidly as those of MP-ROESY, reducing the dephasing ability. Nonetheless, a small gradient throughout the mixing period in CLEANEX-PM is enough to dephase the unwanted magnetization, eliminating the COSY-type peaks (data not shown). It should be noted that, to avoid appreciable signal losses, the gradient strength applied in the mixing period should not be too strong compared to the spin-lock B_1 field. This is because applying a gradient along the z-direction creates a resonance-offset effect, which, in turn, can lessen the spin-lock efficacy at the edge of the sample volume, reducing the available magnetization.

Performance of MP-ROESY and CLEANEX-PM

Figure 5 shows a comparison of Tr-ROESY, MP-ROESY, CLEANEX-PM, CLEANEX-PM II, and conventional ROESY. Using the same gramicidin sample and selective excitation to Phe-NH, the buildup of the Leu $C_\alpha H$ peak was then observed as a function of the mixing time. The buildup rate for each experiments agrees with the theory. MP-ROESY is therefore favorable when the suppression of TOCSY is less demanding and the faster buildup of cross-relaxation peaks is more desirable. CLEANEX-PM and CLEANEX-PM II attenuate the ROE peak intensities to a large extent. The CLEANEX-PM sequence has been successfully applied to detect water–NH exchange in protein, where artifacts from NOE/ROE and TOCSY are eliminated (*18*). The procedure to quantify these exchange rates in a ^{15}N-labeled protein has been presented elsewhere (*42*).

In Fig. 5, CLEANEX-PM data show small ROE intensity at Leu $C_\alpha H$ peak. The reasons may be: (i) the spin-diffusion limit may not hold for this system, so the ROE can slightly dominate; (ii) the off-resonance effect can make the cross-relaxation cancelation imperfect. When the spin-lock transmitter offset was moved from Leu $C_\alpha H$ to an offset midway between Leu $C_\alpha H$ and Phe NH, the ROE peak intensity was further reduced. At shorter mixing time, the ROE intensity is almost within the noise level. Therefore, if exchange occurs at the same peak, it can, at most, only slightly reduce the exchange rate using the initial slope analysis. For intermolecular NOE measurements at longer mixing times, small intramolecular ROE contributions can still exist. Thus, care has to be taken to interpret the data. Changing the spin-lock transmitter offset may be necessary to authenticate the result.

CONCLUSIONS

We have shown several new windowless multiple-pulse sequences that can be used to control and manipulate cross relaxation. The sequences require no special pulse shaping capability. The MP-ROESY experiment is a convenient method for the measurement of cross-relaxation rates for macromolecules. The CLEANEX-PM sequence has a larger bandwidth over which cross-relaxation rates cancel out than competitive sequences. It also has a large scaling factor to inhibit any TOCSY magnetization transfer. This makes it possible to more easily identify the chemical exchange peaks. One distinct advantage of these two new sequences is the simplicity with which they can be implemented, even on older, commercial NMR instruments.

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