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COMMUNICATIONS

Improved Spin-Echo-Edited NMR Diffusion Measurements

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The need for simple and robust schemes for the analysis of ligand-protein binding has resulted in the development of diffusion-based NMR techniques that can be used to assay binding in protein solutions containing a mixture of several ligands. As a means of gaining spectral selectivity in NMR diffusion measurements, a simple experiment, the *g*radient mo*d*ified *s*pin-*e*cho (GOSE), has been developed to reject the resonances of coupled spins and detect only the singlets in the ^{1}H NMR spectrum. This is accomplished by first using a spin echo to null the resonances of the coupled spins. Following the spin echo, the singlet magnetization is flipped out of the transverse plane and a dephasing gradient is applied to reduce the spectral artifacts resulting from incomplete cancellation of the *J*-coupled resonances. The resulting modular sequence is combined here with the BPPSTE pulse sequence; however, it could be easily incorporated into any pulse sequence where additional spectral selectivity is desired. Results obtained with the GOSE-BPPSTE pulse sequence are compared with those obtained with the BPPSTE and CPMG-BPPSTE experiments for a mixture containing the ligands resorcinol and tryptophan in a solution of human serum albumin.

Key Words: **NMR; mixture analysis; diffusion; spin echo.**

INTRODUCTION

A number of innovative approaches that utilize NMR for the study of ligand-protein binding have been reported, including methods that rely on changes in protein chemical shift (*1–3*), relaxation (*4*), transferred NOEs (*5, 6*), saturation (*7*), and diffusion coefficients (*8–12*). The use of diffusion coefficients to distinguish binding from nonbinding ligands offers several advantages in that the approach is rapid, simple, and allows the simultaneous examination of many ligands (*11*).

Several strategies that provide enhanced specificity in NMR diffusion measurements have been presented, facilitating their application to the analysis of complex mixtures. One approach has been to use a ligand labeled with ^{15}N or ^{13}C and employ HSQC- or HMQC-edited diffusion experiments to examine ligand-protein binding (*13, 14*). However, this method has limited utility, as a labeled compound is required. Additional information about a mixture of molecules can be gained by combining the diffusion measurement with a 2D pulse sequence, such as TOCSY or COSY, to provide enhanced selectivity based on spin–spin coupling (*15, 16*). Selectivity for the ligand resonances can be enhanced relative to the spectral background of the protein through subtraction of protein spectra acquired for each gradient amplitude used in the diffusion experiment (*17*). Although effective in elimination of the protein background, this method requires additional experiment time and reduces the signal-to-noise ratio of the subtracted spectra. Relaxation-edited diffusion experiments combining inversion recovery or spin-echo editing with a diffusion pulse sequence have also been used to enhance the selectivity of measurements for complex systems such as protein-ligand mixtures and humic substances (*18–20*). Although spin-echo editing has been demonstrated as an effective method for selecting singlet (resonances without discernable homonuclear spin–spin coupling) magnetization for diffusion measurements, in complex mixtures the residual magnetization from coupled spins can lead to distortions of the integrals of the desired singlet resonances (*18*).

RESULTS AND DISCUSSION

In this communication, a simple strategy is presented for the elimination of residual magnetization arising from coupled spins by modification of the standard spin-echo experiment to produce the *g*radient-mo*d*ified *s*pin-*e*cho (GOSE) pulse sequence shown in Fig. 1a. The delay in the spin-echo portion of the pulse sequence is set to $1/(4J)$ to null the magnetization of the coupled spins. Following the spin echo, a 90° pulse is applied to tip the refocused singlet magnetization to the z axis. Application of a dephasing gradient eliminates the residual magnetization of the coupled resonances and the singlet magnetization is returned to the transverse plane for detection. We have coupled this simple method with the stimulated echo (*21*) incorporating bipolar gradient pulse pairs (BPPSTE) to minimize eddy-current effects and spectral artifacts caused by the gradients (*22*). This sequence, GOSE-BPPSTE, shown in Fig. 1b, is quite effective in selecting

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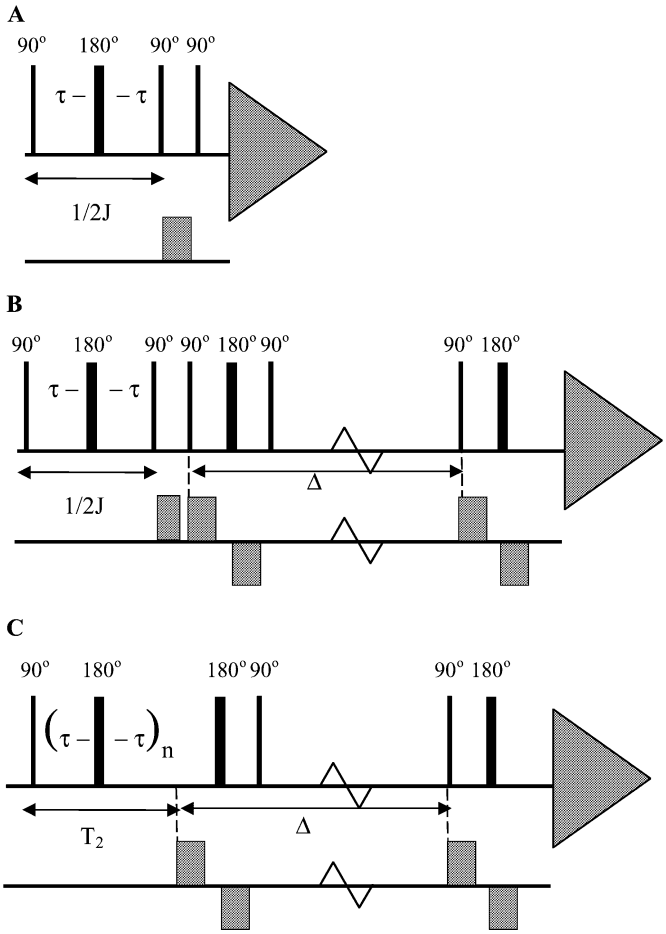

FIG. 1. The GOSE (A), the GOSE-BPPSTE (B), and the CPMG-BPPSTE (C) pulse sequences. A diffusion delay time, Δ, of 0.2 s, a delay following each gradient pulse of 0.01 ms, and a relaxation delay of 1.5 s were used for all experiments. Gradient amplitudes were varied from 2.67 to 24.01 G cm^{-1} and a value of 31 ms was used as the τ delay in the spin-echo portion of the GOSE pulse sequences. The CPMG spectra were acquired using 31 repetitions of the spin-echo pulse train using a τ delay of 1 ms for a total transverse relaxation time of 62 ms.

singlet magnetization in complex spectra, thereby enhancing the spectral selectivity of diffusion measurements.

The effectiveness of the GOSE sequence in eliminating the resonances of the coupled spins can be determined by comparison of the spectra shown in Fig. 2. The one-dimensional spectrum in Fig. 2a was measured for a mixture of the small molecule ligands tryptophan and resorcinol in a solution containing human serum albumin (HSA). The Hahn spin-echo spectrum in Fig. 2b demonstrates that it is possible to use this pulse sequence to select for singlet magnetization using an optimized spin-echo delay time (τ) of 62 ms (*18*). However, substantial artifacts remain due to incomplete elimination of the magnetization of the coupled resonances. These artifacts will be especially problematic as the complexity of the mixture is increased due to the increased likelihood that they will affect the integrated intensity of singlet resonances due to spectral overlap. These artifacts are substantially reduced in the GOSE spectrum, shown in Fig. 2c, as a result of dephasing of the residual magnetization of the coupled spins by the GOSE gradient pulse. Naturally, the effectiveness of this sequence in eliminating the signals from coupled spins depends on the similarity of the homonuclear spin–spin coupling constants of the undesired coupled resonances.

The spectral background produced by the protein can also complicate the analysis of ligand-protein binding with NMR diffusion measurements (*17*). The HSA spectral background makes a noticeable contribution to the BPPSTE spectrum shown in Fig. 3a. However, the protein resonances have been largely eliminated in the GOSE-BPPSTE spectrum of this sample (Fig. 3b). In addition, the artifacts in the GOSE-BPPSTE spectrum have been further reduced from what is observed in Fig. 2b through additional dephasing effects of the BPPSTE sequence. The protein resonances could be attenuated by a combination of the rejection of coupled spins by the GOSE filter and/or by transverse relaxation during the spin-echo period of the pulse sequence. To differentiate between these two possibilities, spectra obtained with the CPMG (Carr–Purcell–Meiboom–Gill)-BPPSTE pulse sequence, shown in Fig. 1c, were also measured (*23, 24*). A similar strategy for coupling a CPMG pulse train to the end

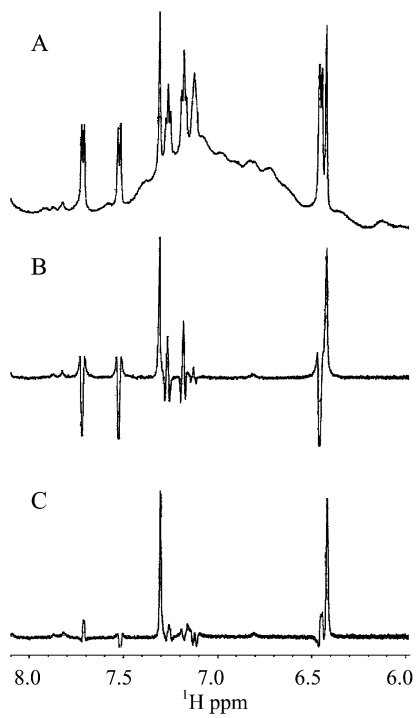

FIG. 2. Spectra of the aromatic region for a HSA, L-tryptophan, and resorcinol mixture using (A) the standard one-dimensional pulse sequence, (B) the Hahn spin-echo, (C) the GOSE pulse sequence. The expected phase distortion of the coupled resonances in (B) is reduced as a result of the 2.67 G cm^{-1} dephasing gradient in (C).

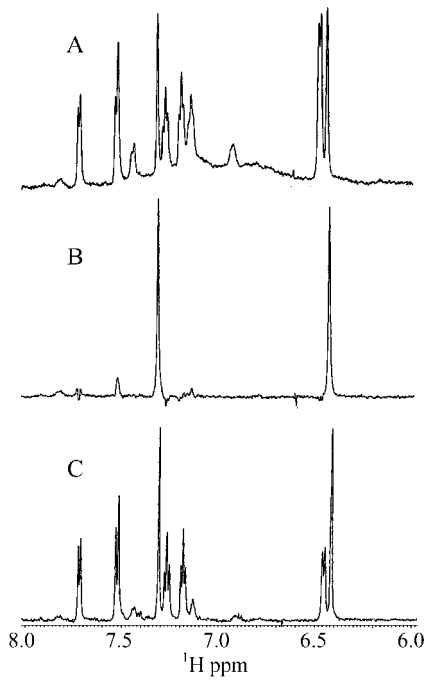

FIG. 3. Spectra of the aromatic region of a HSA, L-tryptophan, and resorcinol mixture using the (A) BPPSTE pulse sequence, (B) the GOSE-BPPSTE sequence, (C) the CPMG-BPPSTE pulse sequence. Spectra were measured with 2.67 G cm^{-1} amplitude gradient pulses in the BPPSTE portion of the pulse sequence.

of the longitudinal eddy current delay diffusion pulse sequence was recently reported for the NOESY analysis of molecules bound to solid-phase synthesis resins (*25*). The spectrum obtained using a 62-ms CPMG pulse train in the CPMG-BPPSTE pulse sequence is shown in Fig. 3c. The reduction of the protein background signal in the CPMG-BPPSTE spectrum compared with the spectrum shown in Fig. 3a indicates that transverse relaxation is the predominate mechanism for attenuation of the protein signals in the GOSE-BPPSTE spectra under the conditions employed here. In cases where the ligand resonances are severely broadened as a result of their interaction with the protein, the GOSE-BPPSTE pulse sequence may also significantly attenuate the desired singlet ligand resonances as a result of T_2 relaxation during the spin-echo delay.

The results obtained for the aromatic tryptophan and resorcinol singlet resonances using the standard and modified BPPSTE pulse sequences are shown in Fig. 4. In this figure the logarithm of resonance intensity is plotted versus gradient amplitude squared for the ligand singlet resonances selected by the GOSE filter, shown in Fig. 3b. Deviations from linearity can result both from spectral overlap between the resonances of different ligands in a complex mixture and from contributions by the protein background to the resonance integrals. The data obtained from spectra acquired with the BPPSTE pulse sequence, shown in Fig. 4a, deviate from a linear fit as a result of spectral overlap of the ligand resonances with those of the much more slowly diffusing HSA. In contrast the data from both the GOSE- (Fig. 4b) and CPMG- (Fig. 4c) filtered BPPSTE spectra produce high-quality straight-line fits due to the reduction of the HSA spectral background. The effect of the HSA magnetization on the diffusion coefficients extracted from the data shown in Fig. 4 can be discerned by comparison of the results. The diffusion coefficients measured with the BPPSTE sequence for both resorcinol and tryptophan are artificially lowered due to overlap with the protein resonances, skewing the diffusion coefficients towards those of the protein. The larger relative deviation observed for the tryptophan resonance at 7.35 ppm is due to the greater HSA intensity in this region of the BPPSTE spectrum. In contrast the results produced by both the GOSE- and CPMG-modified pulse sequences are in good agreement with each other and with the value expected for tryptophan binding at the concentrations employed (*17*).

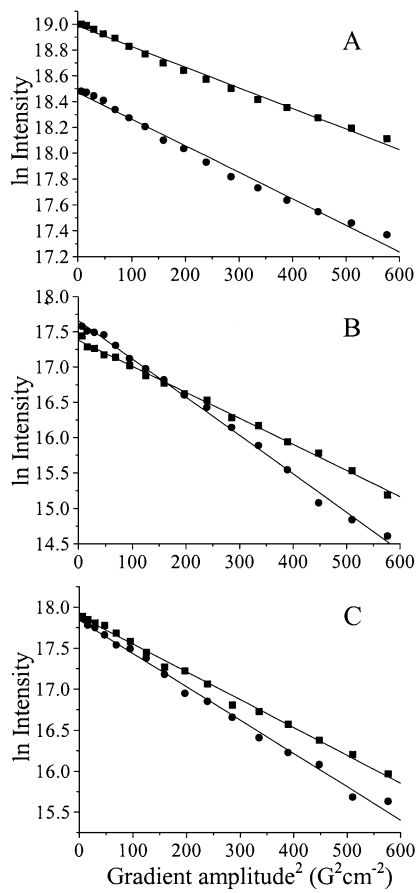

FIG. 4. Results of diffusion experiments utilizing the (A) BPPSTE pulse sequence resorcinol ($2.7 \pm 0.1 \times 10^{-10}$), tryptophan ($2.0 \pm 0.1 \times 10^{-10}$); (B) the GOSE-BPPSTE pulse sequence, resorcinol ($5.9 \pm 0.3 \times 10^{-10}$), tryptophan ($4.4 \pm 0.2 \times 10^{-10}$); and (C) CPMG-BPPSTE pulse sequence, resorcinol ($5.7 \pm 0.3 \times 10^{-10}$), tryptophan ($4.4 \pm 0.2 \times 10^{-10}$); plotted as the natural log of the singlet aromatic resonance intensities for the resorcinol (●, 6.42 ppm) and L-tryptophan (■, 7.35 ppm) versus the gradients amplitude squared. All diffusion coefficients are reported in units of m^2 s^{-1}.

CONCLUSION

We have utilized the GOSE experimental scheme for the measurement of diffusion coefficients for the study of ligand-protein binding; however, this method is applicable to measurements for any complex mixture where simplification based on selection of singlet magnetization is desired. Furthermore, this strategy for the selection of singlet magnetization could be easily incorporated into any standard pulse sequence, for example, NOESY. The effectiveness of this sequence in eliminating the signals from coupled spins in the spectra of complex mixtures is dependent on the similarity of the homonuclear spin–spin coupling constants. Therefore, this sequence should be most effective in selectively observing singlets without interference from the residual magnetization of coupled spins when multiplets all have similar coupling constants, as is the case for the substituted aromatic compounds studied here.

EXPERIMENTAL

All measurements were performed with solutions of 0.3 mM L-tryptophan, 0.3 mM resorcinol, and 0.1 mM human serum albumin (99% agarose electrophoresis, Sigma) in 0.2 M phosphate buffer at pD 7.50. The NMR experiments were performed with a Varian INOVA 600-MHz spectrometer equipped with a 5-mm triple axis gradient probe at 298 K. The amplitude and duration of the dephasing gradient in the GOSE and GOSE-BPPSTE experiments were 2.67 G cm^{-1} and 1 ms, respectively. The gradient pulses in the BPPSTE experiments were 1.2 ms in duration and ranged between 2.67 and 24.01 G cm^{-1}. The spectra were obtained with a spectral window of 9595 Hz by coaddition of 512 transients for the diffusion measurements and 256 transients for all other measurements, using an acquisition time of 0.854 s and a relaxation time of 1.5 s. The FIDs were zero-filled to 32,768 points and multiplied by an exponential function equivalent to 0.5 Hz prior to Fourier transformation.

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