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# Singlet-State Exchange NMR Spectroscopy for the Study of Very Slow Dynamic Processes

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**Abstract:** Singlet states with lifetimes that are longer than spin–lattice relaxation times  $T_S \gg T_1$  offer unique opportunities for studying very slow dynamic processes in solution-state NMR. A set of novel experiments can achieve broadband excitation of singlet states in pairs of coupled spins. The most elaborate of these experiments, two-dimensional singlet-state exchange spectroscopy (SS-EXSY), is independent of the offsets of the two spins, their relative chemical shifts, and their scalar couplings. The new methods open the way to study very slow chemical exchange or translational diffusion using mixing times  $\tau_m \approx T_S \gg T_1$ . The lifetimes  $T_S$  of singlet states of pairs of protons in a partially deuterated saccharide are shown to be longer than the longitudinal proton relaxation times  $T_1$  in the same compound by a factor of ca. 37.

## Introduction

In conventional NMR studies of slow translational diffusion, chemical exchange, and refolding of biomolecules such as proteins or nucleic acids, the upper limit of the accessible time scale is normally determined by the longitudinal relaxation time constant  $T_1$ , which is usually regarded as the maximum lifetime of the memory of nuclear spins. Correlations between states that are connected through very slow processes with a characteristic time constant longer than  $T_1$  could not be monitored by NMR so far. However, recent work by Carravetta and Levitt<sup>1–3</sup> has shown that it is possible to excite and observe so-called singlet states in systems containing pairs of scalar-coupled spins. Such singlet states  $|S_0\rangle = N\{|\alpha\beta\rangle - |\beta\alpha\rangle\}$  with  $N = 2^{-1/2}$  are antisymmetric under a permutation of the two spins, and the singlet-state lifetime  $T_S$  is not affected by the mutual dipole–dipole interaction between these two spins. Singlet-state lifetimes  $T_S$  can be more than an order of magnitude longer than longitudinal relaxation times  $T_1$  in two-spin systems with analogous dynamic properties.<sup>4</sup> A nonvanishing population of a singlet state  $|S_0\rangle$  can be obtained by first exciting a zero-quantum coherence  $ZQ_x$ , i.e., a coherent superposition of two states  $|\alpha\beta\rangle$  and  $|\beta\alpha\rangle$  with a suitable phase, which is then converted into a population of the singlet state  $|S_0\rangle$  by an appropriate radio frequency irradiation that suppresses the effects of the offsets. In practice, this irradiation converts a coupled two-spin IS system ( $J_{IS} \neq 0$ ) into an  $I_2$  system with two

magnetically equivalent spins. Alternatively, as shown below, a singlet state  $|S_0\rangle$  can be populated by first creating a longitudinal two-spin order  $\sigma = 2I_zS_z$ , which, in contrast to a system in thermal equilibrium with  $\sigma = I_z + S_z$ , comprises eigenstates  $|\alpha\beta\rangle$  and  $|\beta\alpha\rangle$  with nonvanishing populations. Like a  $ZQ_x$  coherence, a  $2I_zS_z$  term (also known as “ZZ order”) can be converted into a population of the singlet-state  $|S_0\rangle$  by appropriate radio frequency irradiation, which in effect leads to decoupling of the  $J_{IS}$  interaction. The population of a singlet state can in principle be preserved indefinitely if decoupling is ideal and if all relaxation mechanisms other than the dipolar interaction between spins I and S can be neglected. In practice, nonideal decoupling leads to a reduction in the lifetime  $T_S$ ,<sup>5</sup> as does relaxation of the I and/or S spins by chemical shift anisotropy (CSA) or by dipolar interactions with further spins in the vicinity that may belong to the same molecule or to neighboring (solvent) molecules.

It has been shown that singlet states can be exploited to study slow translational diffusion.<sup>4</sup> Preliminary demonstrations have been carried out with a simple test molecule, 2-chloroacrylonitrile, which contains only two protons I and S with a small difference in chemical shifts  $\Delta\nu_{IS} = \nu_I - \nu_S = 38$  Hz at 300 MHz (0.13 ppm) and a scalar coupling  $J_{IS} = -3$  Hz.<sup>4</sup> We have found that, contrary to earlier belief, proton-containing solvents do not lead to a dramatic reduction of the lifetime  $T_S$  of the singlet states; however, the removal of dissolved oxygen is recommended. Molecules such as saccharides that contain more protons have reduced lifetimes  $T_S$  compared to molecules that contain only isolated proton pairs, but partial deuteration of saccharides in all positions except for the  $H_5'$  and  $H_5''$  protons (we have adopted the numbering appropriate for nucleic acids)

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leaves a pair of diastereotopic protons with  $J_{\text{IS}} = J(\text{H}_5' \text{H}_5'') = -12.5$  Hz and a small difference in chemical shifts  $\Delta\nu_{\text{IS}} = \nu_1 - \nu_{\text{S}} = \nu(\text{H}_5') - \nu(\text{H}_5'') = 75$  Hz at 400 MHz (0.18 ppm). (Such partial deuteration can be achieved conveniently by oxidation of a perdeuterated saccharide to an aldose and subsequent reduction.) When these saccharides are incorporated into nucleic acids such as RNA, the chemical shifts of the  $\text{H}_5'$  and  $\text{H}_5''$  protons should be affected by conformational exchange and refolding processes.<sup>6,7</sup> Such protons can therefore be used to study the kinetics of slow exchange, for example, by two-dimensional exchange spectroscopy (EXSY).<sup>8,9</sup> The kinetic window of such experiments is normally limited by longitudinal relaxation to mixing times  $\tau_{\text{m}} \approx T_1(^1\text{H})$ . In  $^1\text{H}$ -detected  $^{15}\text{N}$  exchange spectroscopy this limitation can be somewhat relaxed since one can use mixing times  $\tau_{\text{m}} \approx T_1(^{15}\text{N}) > T_1(^1\text{H})$ . One of the objectives of this paper is to show that singlet-state (SS) variants of two-dimensional exchange spectroscopy (SS-EXSY) can be designed that extend the observable kinetic window to mixing times  $\tau_{\text{m}} \approx T_{\text{S}}(^1\text{H}) > T_1(^{15}\text{N}) > T_1(^1\text{H})$ .

The pulse sequences for singlet-state excitation that have been described so far<sup>3,4</sup> suffer from a number of drawbacks: (i) the RF carrier  $\nu_{\text{RF}}$  must be positioned halfway between the chemical shifts  $\nu_1$  and  $\nu_{\text{S}}$  of the two nuclei, (ii) the efficiency of the sequences depends on the difference  $\Delta\nu_{\text{IS}} = \nu_1 - \nu_{\text{S}}$  between the chemical shifts, and (iii) the efficiency also depends on the scalar coupling constant  $J_{\text{IS}}$ . Clearly, slow dynamic processes  $\text{A} \leftrightarrow \text{B}$ , such as chemical exchange or refolding of biomolecules like proteins and nucleic acids, must lead to changes in chemical shifts  $\nu_1^{\text{A}} \neq \nu_1^{\text{B}}$  and/or  $\nu_{\text{S}}^{\text{A}} \neq \nu_{\text{S}}^{\text{B}}$  to be observable by NMR. In general, the differences in chemical shifts may also be affected by chemical exchange, i.e.,  $\Delta\nu_{\text{IS}}^{\text{A}} = (\nu_1^{\text{A}} - \nu_{\text{S}}^{\text{A}}) \neq \Delta\nu_{\text{IS}}^{\text{B}} = (\nu_1^{\text{B}} - \nu_{\text{S}}^{\text{B}})$ . Furthermore, it is possible that the scalar couplings are also affected by chemical exchange, i.e.,  $J_{\text{IS}}^{\text{A}} \neq J_{\text{IS}}^{\text{B}}$ . If singlet states are to be used to investigate such slow processes, the pulse sequences must be modified so as to become independent of chemical shifts and couplings.

In this work, we present new methods that make slow exchange phenomena in biological macromolecules amenable to study via singlet-state NMR spectroscopy. To begin with, we introduce ways to excite singlet states in spin pairs with different chemical shifts and  $J$ -coupling constants. Next, we show that adequate broadband decoupling preserves singlet states over a wide range of frequencies, and finally we present a 2D experiment that can correlate signals of coupled spins that are exchanging slowly between different magnetic environments that lead them to have different chemical shifts and  $J$ -couplings. Such slow exchange processes occur, for instance, during the refolding of RNA<sup>7</sup> and play a key role in protein–protein interactions.<sup>10</sup> Their study can greatly benefit from the atomic resolution that NMR can afford in native molecular conditions.

## Theory

As will be shown below, there are two distinct ways of obtaining a system described by a density matrix  $\sigma$  in which

the singlet state  $|S_0\rangle$  has a nonvanishing population  $p(S_0) = \text{Tr}\{\sigma|S_0\rangle\langle S_0|\}$ . Prior to applying the RF field, one must have either a nonvanishing  $\text{ZQ}_x$  zero-quantum coherence or a nonvanishing longitudinal two-spin  $\text{ZZ}$  order  $2I_zS_z$ , or a combination of both  $\text{ZQ}_x$  and  $\text{ZZ}$ . There are two orthogonal forms of zero-quantum coherences, i.e.,  $\text{ZQ}_x = \{|\alpha\beta\rangle\langle\beta\alpha| + |\beta\alpha\rangle\langle\alpha\beta|\}/2$  and  $\text{ZQ}_y = -i\{|\alpha\beta\rangle\langle\beta\alpha| - i|\beta\alpha\rangle\langle\alpha\beta|\}/2$ . Only the  $\text{ZQ}_x$  component can be converted into a singlet state by decoupling. On the other hand, two-spin  $\text{ZZ}$  order can be expressed as a linear combination of four populations, i.e.,  $2I_zS_z = \{|\alpha\alpha\rangle\langle\alpha\alpha| - |\alpha\beta\rangle\langle\alpha\beta| - |\beta\alpha\rangle\langle\beta\alpha| + |\beta\beta\rangle\langle\beta\beta|\}/2$ . Only the middle two terms can contribute to the population of the singlet state  $p(S_0)$  when the RF irradiation is switched on. This population  $p(S_0)$  can be determined by converting the  $\text{ZQ}_x$  and  $\text{ZZ}$  terms contained in the density operator from the product base PB into the singlet–triplet base STB. Likewise, when the RF irradiation is switched off at the end of the mixing interval  $\tau_{\text{m}}$ , it is sufficient to convert the remaining population  $p(S_0)$  from the singlet–triplet base STB into  $\text{ZQ}_x$  and  $\text{ZZ}$  terms in the product base PB. The latter can then be converted into observable terms following the usual rules of product operator calculations.<sup>11</sup>

Through a simple basis transformation, any Cartesian operator product represented in the product base PB of the weakly coupled IS system can be expressed in the singlet–triplet base STB of the  $I_2$  system when the two spins are in effect made to be magnetically equivalent. The matrix representations of various terms in the two bases are given in Table 1 of the Supporting Information. The following combinations of Cartesian operator terms in the product base PB can contribute to the populations of the singlet–triplet base STB:

$$\{E, (I_z + S_z), 2I_zS_z, \text{ZQ}_x\}_{\text{PB}} \quad (1)$$

The conversion matrix is the following:

$$\begin{pmatrix} |S_0\rangle\langle S_0| \\ |T_0\rangle\langle T_0| \\ |T_1\rangle\langle T_1| \\ |T_{-1}\rangle\langle T_{-1}| \end{pmatrix} = \begin{pmatrix} \frac{1}{4} & 0 & -\frac{1}{2} & -1 \\ \frac{1}{4} & 0 & -\frac{1}{2} & 1 \\ \frac{1}{4} & \frac{1}{2} & \frac{1}{2} & 0 \\ \frac{1}{4} & -\frac{1}{2} & \frac{1}{2} & 0 \end{pmatrix} \cdot \begin{pmatrix} E \\ I_z + S_z \\ 2I_zS_z \\ \text{ZQ}_x \end{pmatrix} \quad (2)$$

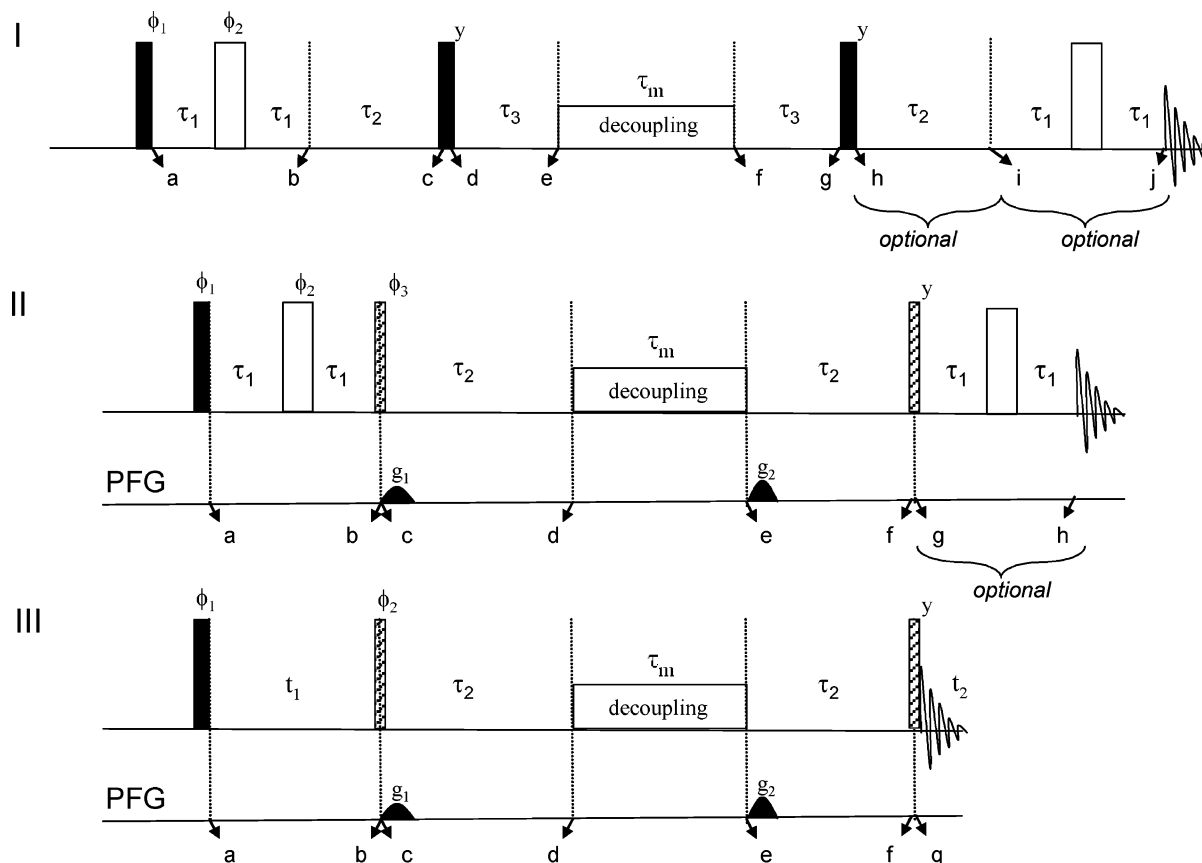
It can readily be seen that only longitudinal two-spin order  $2I_zS_z$  and real zero-quantum coherence  $\text{ZQ}_x$  can be converted into singlet-state populations. The inverse transformation can be described by the following matrix:

$$\begin{pmatrix} E \\ I_z + S_z \\ 2I_zS_z \\ \text{ZQ}_x \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 0 & 0 & 1 & -1 \\ -\frac{1}{2} & -\frac{1}{2} & \frac{1}{2} & \frac{1}{2} \\ -\frac{1}{2} & \frac{1}{2} & 0 & 0 \end{pmatrix} \cdot \begin{pmatrix} |S_0\rangle\langle S_0| \\ |T_0\rangle\langle T_0| \\ |T_1\rangle\langle T_1| \\ |T_{-1}\rangle\langle T_{-1}| \end{pmatrix} \quad (3)$$

Clearly, if we ignore the unobservable unity operator  $E$ , the singlet-state populations can only be converted into longitudinal two-spin order  $2I_zS_z$  and real zero-quantum coherence  $\text{ZQ}_x$ . The transformations of the 16-dimensional Liouville space are given in the Supporting Information.

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**Figure 1.** Pulse sequences designed for excitation of singlet-state populations and their subsequent reconversion into observable magnetization. Pulses with flip angles of  $\pi$ ,  $\pi/2$ , and  $\pi/4$  ( $45^\circ$ ) are indicated by open, filled, and hatched rectangles, respectively. All pulses are applied with phases along the  $x$ -axis of the rotating frame, unless otherwise indicated. The delays must be adjusted to the offsets  $\nu_I$  and  $\nu_S$  of spins I and S and to the coupling constant  $J_{IS}$  according to Table 1:  $\tau_1 = 1/[4J_{IS}]$ ,  $\tau_2 = 1/[2(\nu_I - \nu_S)]$ ,  $\tau_3 = \tau_2/2$ . Sequence I also requires that the carrier be set halfway between the two chemical shifts,  $\nu_{RF} = (\nu_I + \nu_S)/2$ . (I) Sequence for singlet-state excitation designed by Caravetta and Levitt,<sup>3</sup> expanded with some optional delays for the sake of symmetry. The singlet state is excited only via ZQ coherences which precess in the  $\tau_3$  intervals. The phase cycle is  $\varphi_1 = x, -x$ ,  $\varphi_2 = 2(x), 2(-x)$ , and  $\varphi_{rec} = x, -x$ . (II) Sequence for singlet-state excitation that uses both ZQ coherences and longitudinal two-spin order  $2I_zS_z$  (“ZZ” order) in the  $\tau_2$  intervals. The phase cycle was  $\varphi_1 = x, -x$ ,  $\varphi_2 = 2(x), 2(-x)$ ,  $\varphi_3 = 4(y), 4(-y)$ , and  $\varphi_{rec} = 2(x, -x), 2(-x, x)$ . (III) Sequence designed for two-dimensional singlet-state exchange spectroscopy (2D SS-EXSY) to monitor correlations between different states of a spin system undergoing a slow dynamic process. The singlet state is excited via a combination of ZQ and ZZ terms in the  $\tau_2$  intervals. The phase cycle was  $\varphi_1 = x, -x$ ,  $\varphi_2 = 2(y), 2(-y)$ , and  $\varphi_{rec} = x, 2(-x), x$ . Quadrature detection in the  $t_1$  dimension was achieved by time-proportional phase incrementation of  $\varphi_1$  (TPPI). The pulsed-field gradients (PFG)  $g_1$  and  $g_2$  lead to dephasing of coherences with order  $p \neq 0$ .

## Experimental Methods

This study presents a series of new spectroscopic methods that are designed to fulfill the following demands:

(i) Broadband excitation of singlet-state populations in both 1D and 2D NMR spectroscopy to convert the equilibrium populations of the IS system into singlet-state populations of the  $I_2$  system over a wide range of shifts and couplings. Previous methods contained intervals that had to be adapted to the coupling constants and to the offsets between the individual chemical shifts and the RF carrier, which made them impractical for molecules containing coupled spin pairs featuring a range of chemical shifts or couplings;

(ii) An evaluation of adequate broadband composite decoupling methods for optimizing the lifetime  $T_S$  of the singlet state when there is a nonvanishing offset between the average chemical shift of the two spins and the RF carrier;

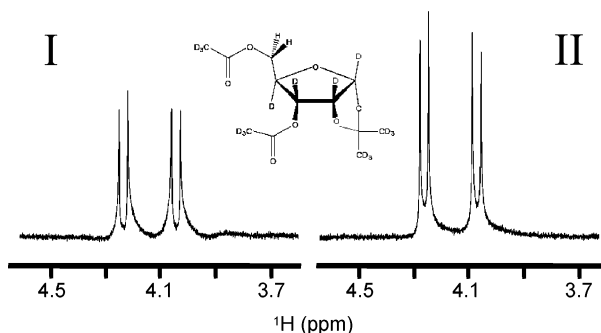
(iii) Two-dimensional experiments designed to study correlations between different environments, with a mixing time period  $\tau_m$  where the “memory” of the system is “stored” in the form of singlet-state populations.

Figure 1 shows a comparison between existing methods (that require prior knowledge of the chemical shifts and scalar couplings) and new proposed techniques for broadband singlet-state excitation. A step-by-step analysis of the sequences in terms of product operators is given in

the Supporting Information. In sequence I of Figure 1 (which becomes equivalent to the experiment of Caravetta and Levitt if the optional delays at the end are dropped), single-quantum in-phase coherences excited at point a are transformed into antiphase coherences between points a and b. Between points b and c, the chemical shifts lead to a conversion of the real terms  $2I_zS_x + 2I_xS_z$  (which have the same phase) into imaginary terms  $2I_zS_y - 2I_yS_z$  (with opposite phases). To achieve this effect, the carrier  $\nu_{RF} = (\nu_I + \nu_S)/2$  has to be set precisely halfway between the chemical shifts of spins I and S. The  $(\pi/2)_y$  pulse at point c excites imaginary zero-quantum coherence  $ZQ_y = 2I_xS_y - 2I_yS_x$ . Between points d and e, this is converted into the desired real  $ZQ_x$  coherence. As explained in more detail in the Supporting Information, this coherence is transformed into opposite contributions to the populations of the singlet and central triplet states under RF irradiation starting at point e. The triplet state populations can be neglected because they do not survive the protracted decoupling period.

The new sequence II in Figure 1 was designed for broadband excitation of singlet states in coupled spin systems. In contrast to sequence I, where the carrier must be set halfway between the two chemical shifts, i.e.,  $\nu_{RF} = (\nu_I + \nu_S)/2$ , sequence II does not have any requirement on  $\nu_{RF}$ . Consequently, it is possible to study molecules with multiple sites undergoing dynamic effects if they feature different chemical shifts in the two conformations A and B, provided the





**Figure 2.** Comparison of signals of the  $H_5'$  and  $H_5''$  protons in the partly deuterated saccharide shown in the inset, obtained with sequences I (left panel) and II (right panel) of Figure 1, including the “optional” elements, at 300 K and 400 MHz. Sequence II is less prone to various relaxation effects. The doublets stem from the diastereotopic protons with  $J(H_5', H_5'') = -12.5$  Hz. The fixed intervals were  $\tau_1 = 20$  ms,  $\tau_2 = 6.7$  ms, and  $\tau_m = 5$  s. The continuous decoupling field had an amplitude of ca. 1.2 kHz. To limit artifacts arising from convection induced by sample heating, a Shigemitsu sample tube was used with a sample height of ca. 15 mm.

chemical shift differences between the coupled pairs of protons are preserved ( $\Delta\nu_{IS}^A = \Delta\nu_{IS}^B$ ). This is achieved by exciting both  $ZQ_x$  coherence and longitudinal two-spin order  $2I_zS_z$  using a  $(\pi/4)_x$  pulse at point b. Between time points c and d, the sign of the  $ZQ_x$  coherence is reversed ( $ZQ_x \rightarrow -ZQ_x$ ) under the effect of the difference of the chemical shifts  $\Delta\nu_{IS} = \nu_1 - \nu_S$ , a process which we may refer to as “ZQ reversal”. Since  $\tau_2 = 1/(2\Delta\nu_{IS})$ , this requires prior knowledge of the relative chemical shifts but not of the individual offsets. This ZQ reversal is necessary to prevent mutual cancellation of ZQ and ZZ contributions to the singlet state. As seen from the analysis provided in the Supporting Information, the singlet population created at point d of sequence II is equal to the singlet population created at point e of sequence I. The sensitivities of these two experiments are therefore comparable if losses through various relaxation processes can be neglected. It should be emphasized that the mechanisms are not the same: in sequence I, only a  $ZQ_x$  term contributes to the population of the singlet state, while a combination of  $ZQ_x$  and  $2I_zS_z$  contributes in sequence II. In the last part of sequence II, the singlet-state population remaining at the end of the mixing time  $\tau_m$  is reconverted into single-quantum coherences via symmetrical processes. If the optional delays at the end of sequence II are used, two in-phase doublets with the same sign for both spins I and S can be observed. In Figure 2 we show a comparison between signals detected using sequences I and II in Figure 1, using the pair of protons in a partly deuterated saccharide shown in the inset.

The 2D sequence III in Figure 1 does not contain any fixed  $[\tau_1 - \pi - \tau_1]$  interval for generating antiphase magnetization, since such terms will build up naturally, with a coefficient  $\sin(\pi J_{IS}t_1)$ , in the course of the evolution time  $t_1$ . Like other 2D experiments with antiphase multiplet structures (such as COSY, ZZ-EXSY, etc.), this requires an adequate choice of the maximum duration  $t_1^{\max}$  of the evolution period so that one achieves sufficient resolution in the  $\omega_1$  domain to resolve the antiphase  $J$ -coupled multiplets. In sequence III, the coherence transfer from both I and S spins that undergo precession in the evolution period contributes to both ZQ and ZZ terms and, hence, to the population of the singlet state during the mixing time. The reversion of singlet-state populations into ZQ and ZZ terms and from there into observable (antiphase) coherences of spins I and S follows similar pathways as described above for sequence II.

It is noteworthy that in the case where not only the average chemical shift but also the difference  $\Delta\nu_{IS} = \nu_1 - \nu_S$  changes from site to site ( $\Delta\nu_{IS}^A \neq \Delta\nu_{IS}^B$ ) neither is it possible to ensure that the  $ZQ_y$  terms in sequence I are converted into  $ZQ_x$ , nor can one be sure that the  $ZQ_x$  components in sequences II or III are converted into  $-ZQ_x$ . Therefore,

it is preferable to suppress the zero-quantum terms by a filter proposed by Thrippleton and Keeler,<sup>12,13</sup> since the longitudinal two-spin order  $2I_zS_z$  can still be used to generate singlet-state populations. This is demonstrated in the 1D and 2D sequences IV and V of Figure 3, where two frequency-swept pulses and pulsed field gradients, both of duration  $\tau_f$ , are inserted prior to and after the mixing period. The most effective suppression of  $ZQ_x$  was achieved by using a first gradient along the  $z$ -axis and a second gradient along an orthogonal ( $x$  or  $y$ ) axis. The use of the filter makes the efficiency of singlet-state excitation and reconversion entirely independent not only on the chemical shift differences  $\Delta\nu_{IS} = \nu_1 - \nu_S$  but also on the individual offsets  $\nu_1$  and  $\nu_S$ . This extension of the range comes at a cost of 50% of the signal intensity for each interval where the ZQC is suppressed, so the 2D sequence V affords only 25% of the signal detected with the 2D sequence III (see Supporting Information). The pathways shown below the 2D sequence of Figure 3 indicate the population transfer processes that occur between the evolution and detection intervals.

In systems where the selective excitation of ZZ order suffices, say to determine the time constant  $T_S$  in the absence of exchange, it is possible to excite ZZ order directly from thermal equilibrium by selective inversion of one or several lines in the single-quantum spectrum<sup>14</sup> albeit at the expense of a 50% loss in signal intensity which can only be recovered by using both ZZ and ZQ terms.

Table 1 summarizes to what extent the fixed delays in sequences I to V have to be adapted to the scalar coupling constant  $J_{IS}$  and to the difference in chemical shifts  $\Delta\nu_{IS} = \nu_1 - \nu_S$ . Only the original sequence I of Carravetta and Levitt<sup>1</sup> requires that the RF carrier be positioned in the center of the spectrum  $\nu_{RF} = (\nu_1 + \nu_S)/2$ . If there are no requirements, the sequences may be considered to be *broadband* with respect to the average shift  $(\nu_1 + \nu_S)/2$ , to the relative shift  $(\nu_1 - \nu_S)$ , and to the coupling constant  $J_{IS}$ .

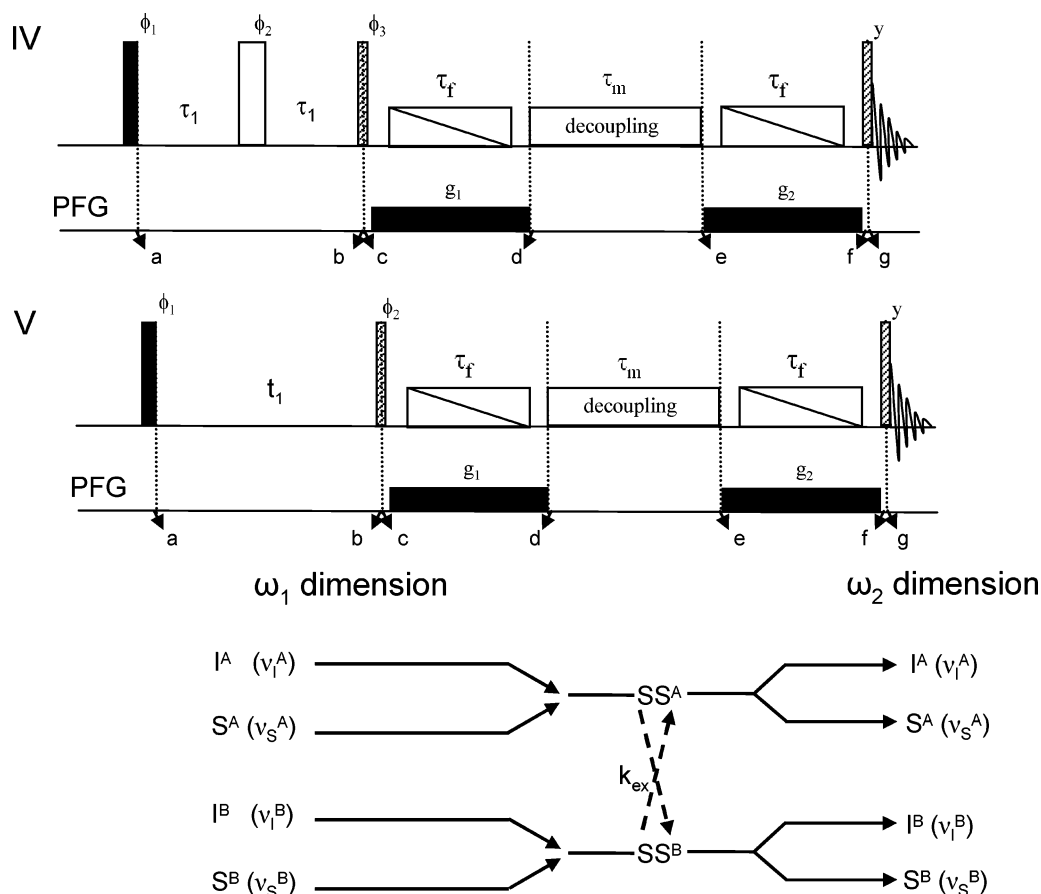
The broadband character of the excitation and reconversion of singlet states is not the only requirement for studying pairs of spins in different environments simultaneously. In order to preserve the populations of singlet states for long time periods, suitable decoupling sequences have to be used. Figure 4 shows signals of the saccharide described below, observed using sequence II while stepping the offset between the RF carrier and the average chemical shift, using either continuous-wave (CW) decoupling or composite-pulse radio frequency irradiation. The use of the WALTZ16 sequence<sup>15</sup> has proven to be effective in preserving the singlet state over a range of  $\pm 1$  kHz with respect to the carrier ( $\pm 2.5$  ppm at 400 MHz). This is a satisfactory range for typical groups involving coupled protons in biomolecules, such as diastereotopic  $CH_2$  groups in proteins or nucleic acids.

A mixing period  $\tau_m$  with continuous decoupling, no matter how weak, may lead to undesirable heating effects that can cause convection currents. This phenomenon can lead to a shortening of the apparent lifetimes  $T_S^{\text{app}} < T_S$  of the singlet-state populations if the molecules that carry these singlet states move out of the sensitive volume enclosed by the transmitter and receiver coils. The use of Shigemitsu tubes with a limited sample height is recommended to combat such artifacts.

## Results and Discussion

A monosaccharide with a five-membered furanose ring was synthesized in such a manner that all hydrogen atoms except those in positions  $H_5'$  and  $H_5''$  were substituted by deuterium atoms. The  $H_5'$  and  $H_5''$  protons have a chemical shift difference of 75 Hz at 400 MHz (0.18 ppm) and a scalar coupling constant

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**Figure 3.** (IV) Sequence designed for 1D spectroscopy with excitation of singlet-state populations only via two-spin ZZ order  $2I_S S_z$ , using filters to suppress the ZQ terms in the  $\tau_f$  intervals. The rectangles with diagonal bars represent adiabatic frequency-swept pulses applied in conjunction with pulsed field gradients (black rectangles) to suppress zero-quantum coherences. The phase cycle is the same as that for sequence II. (V) Sequence for 2D SS-EXSY using only two-spin ZZ order. The phase cycle is the same as that for sequence III. The chemical shift correlations that can be observed in the 2D spectra are shown schematically below the pulse sequence.

**Table 1.** Comparison of the Five Sequences Presented in Figures 1 and 3, Showing Which Delays Must Be Adapted to the Coupling Constant  $J_{IS}$ , the Relative Shift  $(\nu_I - \nu_S)$ , and the Average Shift  $(\nu_I + \nu_S)/2^a$

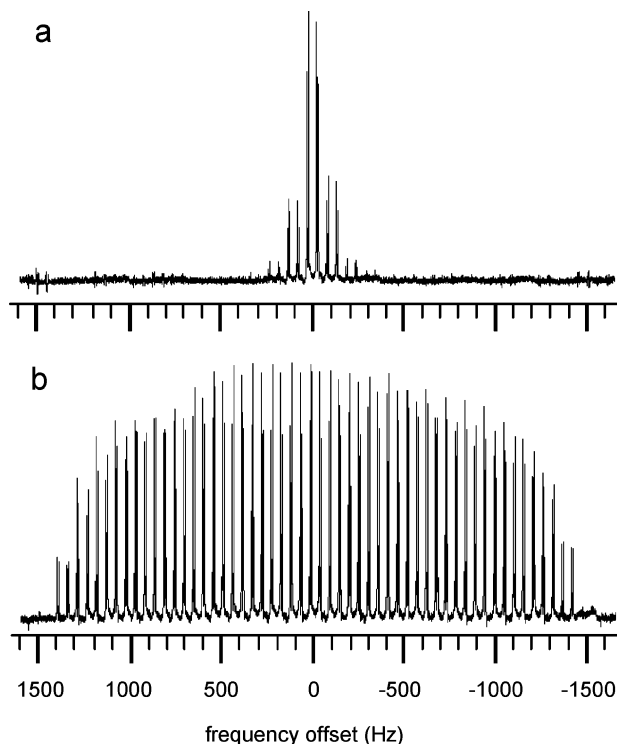
adjustment with respect to	sequence I	sequence II	sequence III	sequence IV	sequence V
coupling constant $J_{IS}$	$\tau_1 = 1/ 4J_{IS} $	$\tau_1 = 1/ 4J_{IS} $	broadband	$\tau_1 = 1/ 4J_{IS} $	broadband
relative shift $\Delta\nu_{IS} = \nu_I - \nu_S$	$\tau_2 = 1/(2\Delta\nu_{IS})$	$\tau_2 = 1/(2\Delta\nu_{IS})$	$\tau_2 = 1/(2\Delta\nu_{IS})$	broadband	broadband
average shift $(\nu_I + \nu_S)/2$	$\tau_3 = \tau_2/2$	broadband	broadband	broadband	broadband
relative sensitivity (%) (neglecting relaxation)	100	100	100	25	25

<sup>a</sup> If there is no need for any adjustment, the experiment has a broadband character with respect to the corresponding parameters.

of  $J_{IS} = J(H_5'H_5'') = -12.5$  Hz. The sample was initially dissolved in deuterated degassed DMSO in view of reducing intermolecular dipolar interactions with the solvent. For both  $H_5'$  and  $H_5''$  protons, we determined the same nonselective longitudinal relaxation time constant  $T_1(H_5') = T_1(H_5'') = 0.7 \pm 0.03$  s, while the lifetime of the singlet state was found to be  $T_S(H_5', H_5'') = 26 \pm 3$  s, which is longer than  $T_1$  by a factor of  $37 \pm 6$ . In nondeuterated degassed DMSO, we have measured a similar value. This shows that the long lifetimes of singlet states can be exploited in protonated solvents. Removal of paramagnetic oxygen by using a few pump–freeze–thaw cycles or, as in our case, by letting a stream of argon bubble through the sample is essential to obtain the maximum value of  $T_S(H_5', H_5'')$ , which otherwise can be as low as 15 s. It is however difficult to seal degassed samples while using sample tubes with

a limited height to combat convection. For a similar saccharide where none of the protons were substituted by deuterons, we measured  $T_1(H_5') = T_1(H_5'') = 0.34 \pm 0.02$  s and  $T_S(H_5', H_5'') = 1.5 \pm 0.2$  s. Thus, partial deuteration leads to a 2-fold extension of the longitudinal relaxation time constant  $T_1$  and to a 17-fold extension of the singlet-state lifetime  $T_S$ .

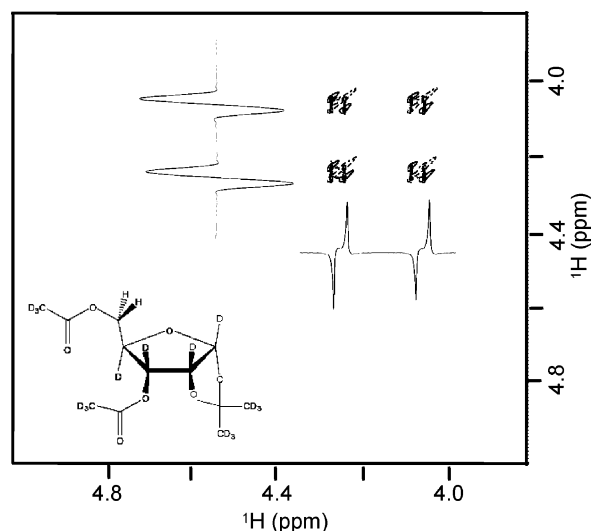
Figure 5 shows a two-dimensional singlet-state exchange (SS-EXSY) spectrum of the selectively deuterated monosaccharide, recorded using sequence III of Figure 1. The 2D spectrum was deliberately acquired while placing the carrier 160 Hz away from the average shift of the two proton spins, to illustrate the broadband character of the method. The mixing time during which spins were maintained in the singlet state was  $\tau_m = 12$  s. As expected, each spin gives rise to antiphase doublets in both dimensions with respect to the  $J$  coupling.



**Figure 4.** Signal intensities (obtained with sequence II) of the partly deuterated saccharide shown in Figure 2, as a function of the offset  $\Delta\nu = \nu_{av} - \nu_{RF}$  between the center of the two resonances  $\nu_{av} = (\nu_1 + \nu_S)/2$  and the radio frequency carrier  $\nu_{RF}$ . (a) CW decoupling; (b) WALTZ16 decoupling. The frequency  $\nu_{RF}$  was used for all pulses and during the decoupling period, and it was incremented in 33 steps of 100 Hz. The amplitude of the decoupling field was  $\nu_1 = \omega_1/2\pi = 976$  Hz, and the duration of the mixing period was  $\tau_m = 10$  s. The fixed intervals were the same as those in Figure 2. At each step a signal consisting of two in-phase doublets is detected, as in Figure 2.

This 2D experiment is conceived to excite singlet states in different environments (for example in two distinct environments A and B where the spins I and S have chemical shifts and couplings  $\nu_1^A$ ,  $\nu_S^A$ ,  $J_{IS}^A$  and  $\nu_1^B$ ,  $\nu_S^B$ ,  $J_{IS}^B$ , respectively). The chemical shifts are first labeled in the evolution interval  $t_1$ , so that the coherences at point b in sequences III and V are modulated at the frequencies  $\nu_1^A$ ,  $\nu_S^A$ ,  $\nu_1^B$ , and  $\nu_S^B$ . Singlet states  $SS^A$  and  $SS^B$  are then excited at point d for both environments A and B. During the interval  $\tau_m$ , exchange may occur between the environments A and B, leading to a partial interconversion of singlet-state populations  $SS^A$  and  $SS^B$ . At point g the singlet-state populations are transformed back into antiphase single-quantum coherence of spins I and S and resume precession at the four chemical shifts in the  $t_2$  detection interval. Thus, even in the absence of exchange, a magnetization that was initially labeled at a frequency  $\nu_1^A$  during  $t_1$  is detected at frequencies  $\nu_1^A$  and  $\nu_S^A$  in  $t_2$  because the spins I and S are mixed to form a common singlet state during  $\tau_m$ . If exchange between states A and B occurs, additional signals will be detected at frequencies  $\nu_1^B$  and  $\nu_S^B$  in  $t_2$ , as shown schematically in Figure 3. Thus, in the presence of exchange, one diagonal and three cross-peak multiplets can be detected at each  $\omega_1$  frequency. In the absence of exchange, only one diagonal and one cross-peak multiplet (which arises from the blending of the magnetization of two the spins in the singlet state) are detected, as it can be seen in Figure 5.

The structure of a 2D spectrum obtained using the sequence in Figure 3 is reminiscent of that of ZZ-EXSY<sup>14</sup> spectra, so, by



**Figure 5.** 2D singlet-state exchange spectrum of the  $H_5'$  and  $H_5''$  protons in the partly deuterated saccharide again shown in the inset, recorded with sequence III at 300 K and 400 MHz. Positive and negative signals are represented by continuous and dotted contours. A matrix of  $32k \times 256$  points was acquired and transformed to  $32k \times 1k$  points. The spectral widths in the  $\omega_2$  and  $\omega_1$  dimensions were 10.2 and 2.6 ppm (ca. 4 and 1 kHz). The carrier frequency  $\nu_{RF}$  was set at 4.5 ppm, at an offset  $\Delta\nu = \nu_{av} - \nu_{RF} = 160$  Hz from the center of the two resonances  $\nu_{av} = (\nu_1 + \nu_S)/2$ . The duration of the mixing period with WALTZ16 decoupling was  $\tau_m = 12$  s, and the amplitude of the decoupling field was 976 Hz. The fixed intervals were the same as those in Figure 2. The peak amplitudes of the  $g_1$  and  $g_2$  pulsed field gradients (PFG) were 73% and 29% of the maximum intensity (50 G/cm), respectively.

analogy, we like to refer to our new singlet-state method as SS-EXSY. The side chain of Tyrosine 35 in basic pancreatic trypsin inhibitor (BPTI) is a good illustration for exchange studies, as the shifts of the four protons in the  $\delta$  and  $\epsilon$  positions are well-dispersed.<sup>16</sup> Chemical exchange ( $H^{\delta 1} \leftrightarrow H^{\delta 2}$  and  $H^{\epsilon 1} \leftrightarrow H^{\epsilon 2}$ ) appears due to the slow rotation ( $\sim 30$  s<sup>-1</sup> at 309 K) of the Tyr ring around the  $C^\beta$ – $C'$  axis. The singlet state can be created for coupled pairs of protons on both sides of the ring (i.e., the pairs  $H^{\delta 1}$ – $H^{\epsilon 1}$  and  $H^{\delta 2}$ – $H^{\epsilon 2}$ , both with a  $J$ -coupling constant of  $\sim 8$  Hz). A 2D SS-EXSY spectrum of the aromatic region of BPTI is shown in Figure 6. As with ZZ exchange spectroscopy<sup>14</sup> four cross-peak multiplets of Tyr35 were observed centered on the chemical shift position of  $H^{\delta 2}$  in the  $\omega_1$  domain, at the chemical shifts of the following protons in the  $\omega_2$  domain:  $H^{\delta 2}$  (diagonal peak, coded in green in Figure 6),  $H^{\epsilon 2}$  (due to the mixing of magnetization of the two spins in the singlet state, in blue), and  $H^{\epsilon 1}$  and  $H^{\delta 1}$  (both of which are due to slow exchange, color-coded in red). For residues Y10, Y21, and Y23 in BPTI, the exchange cross-peaks coincide with the singlet-mixing cross-peaks, due to the fact that the chemical shifts of the  $H^{\delta 1}$  and  $H^{\delta 2}$  protons, on the one hand, and those of the  $H^{\epsilon 1}$  and  $H^{\epsilon 2}$  protons, on the other hand, are averaged by fast exchange on the NMR time scale.

The 2D method illustrated by the spectra of Figures 5 and 6 would prove useful if it can be applied to the study of RNA strands that slowly interconvert between different conformations. In a recent study, an RNA 34-mer was shown to exist in three conformations. The rates of the slow exchange processes suggest that refolding involves the simultaneous dissociation of several base pairs. When slowly relaxing longitudinal <sup>15</sup>N-magnetization

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