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Long-lived States in Multiple-Spin Systems

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Slow dynamic processes, such as folding or unfolding of proteins, can be witnessed by nuclear spins, provided that non-equilibrium populations of the energy levels can be sustained over time intervals that are on the order of the inverse of the rate constants of the dynamic processes. Large biomolecules have slow diffusion rates and may host exchange processes that involve high-energy barriers, such as concerted breaking and making of multiple hydrogen bonds,[1] or isomerisation in proline residues.[2]

The lifetimes of the populations of ordinary Zeeman eigenstates, i.e., the well-known longitudinal relaxation times T_1, are limited by various anisotropic interactions that are modulated by molecular tumbling. For spins with $I=1/2$, the dominant fluctuating interactions are dipole–dipole couplings and anisotropic chemical shifts. An obvious way to limit these magnetic interactions, and thereby extend the lifetimes of coherences, is to use spins with low gyromagnetic ratios ('heteronuclei'),[1] such as nitrogen-15. Methods have been developed to use heteronuclei in deuterated samples for resonance assignment,[3–5] opening the way for structural studies of paramagnetic proteins,[6] protein–protein interactions,[7] intrinsically disordered proteins,[8] slow diffusion,[9] protein dynamics,[10] and slow exchange. A more elegant approach is based on 'long-lived states' (LLS), which in isolated two-spin systems are immune to relaxation mechanisms that are symmetric with respect to spin-exchange.[11,12] It was demonstrated that such states can be excited by appropriate pulse sequences and sustained either by a suitable radio-frequency (rf) irradiation or by moving the sample out of the static field. The populations of these states have relaxation time constants T_{LLS} that can be much longer than T_1. Ratios as large as $T_{LLS}/T_1=37$ have been observed in partly deuterated saccharides. The longest lifetime recorded so far is $T_{LLS}=1583$ s $=26$ min ($T_{LLS}/T_1=8$), observed[13] in $^{15}N_2O$, using LLS involving 'heteronuclei'. The resulting long-lived states offer very useful tools to follow slow exchange,[14] flow or diffusion.[15,16] Applications of long-lived states should become increasingly widespread provided they can be excited in systems featuring diverse spin patterns with more than two coupled spins. Alternatively, since the main (dipolar) interaction is silenced only for the pair of spins participating in the antisymmetric state, extended lifetimes can be obtained by substituting all remaining non-participating protons within a sphere of ca 5 Å by deuterons.[17]

Significant progress has been made towards extending the range of applications of LLS. Long lifetimes have been obtained in symmetric molecules comprising four coupled spins. Thus, Pileio et al. measured ratios $T_{LLS}/T_1=8$ in citric acid.[18] Pileio et al.[19] have also shown how the eigenvalues of the Liouvillian can be analysed to identify long-lived states by diagonalisation, also proposed by Gopalakrishnan et al.[20] This method is reviewed below. In particular, it has been found that long-lived states can be excited and preserved in high fields in AA'BB' systems or in two-spin AM systems in presence of a third spin X.[18,19] A theoretical analysis of the molecular geometries of systems that can sustain states with long lifetimes has been given by Vinogradov and Grant.[21,22] Some LLS can be enhanced by magnetisation transferred from *para*-hydrogen.[23,24]

We have recently found that slow diffusion rates of biomolecules may be determined with moderate gradient strengths. Long-lived states can be excited and sustained for the diastereotopic pairs of protons $\{H^{\alpha1}, H^{\alpha2}\}$ in glycine residues.[25] It appears of interest to design methods to excite long-lived states in other amino-acids. In this paper, we analyse spin states with long lifetimes that can be excited and sustained in some common amino-acids such as serine, cysteine, aspartic acid, asparagine, and histidine, where the aliphatic protons H^{α}, $H^{\beta1}$, and $H^{\beta2}$ form coupled three-spin systems, as well as in acrylic acid, glycerol, and taurine, an important marker of brain function. Once the possibility of quenching relaxation by exciting suitable spin states in an ensemble of nuclei by carefully-designed radio-frequency (rf) pulse sequences has been demonstrated, we can hope to extend this benefit to more general systems.

An effective way of identifying long-lived states in arbitrary spin systems is based on the diagonalisation of their average Liouvillians.[19,20] These incorporate the static and time-dependent parts of the Hamiltonians, and the latter include both rf pulse sequences designed to sustain the LLS (notably by suppressing the chemical shifts to enforce magnetic equivalence) and the fluctuating random anisotropic DD and CSA interactions that lead to relaxation. By diagonalising the Liouvillian, the longest-lived states can be identified as eigenstates corresponding to (negative and real) eigenvalues having the smallest absolute values.

Since both coherent and dissipative terms are included in the Liouvillian, the structure of the longest-lived states depends on spectral parameters such as chemical shifts and *J*-couplings, as well as on the molecular geometry which determines internuclear distances and CSA tensors. A long-lived

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state (LLS) in a system comprising two *J*-coupled spins *I* and *S* can be expressed as Equation (1):[17,26]

$$Q_{\mathrm{LLS}}^{\mathrm{IS}} = -\frac{2}{\sqrt{3}}\left(I_{\mathrm{x}}S_{\mathrm{x}} + I_{\mathrm{y}}S_{\mathrm{y}} + I_{\mathrm{z}}S_{\mathrm{z}}\right) = -\frac{2}{\sqrt{3}}\vec{I}\cdot\vec{S} \tag{1}$$

where the negative sign indicates that this operator corresponds to the difference between the populations of singlet and triplet states. This type of long-lived state can be created in glycine, where $I = \mathrm{H}^{\alpha 1}$ and $S = \mathrm{H}^{\alpha 2}$ are diastereotopic and hence chemically inequivalent, provided the residue is part of a stereogenic peptide or protein. A ratio as high as $T_{\mathrm{LLS}}/T_1 = 25$ has been found in the Ala-Gly dipeptide.[25]

One of the three spin systems studied herein, acrylic acid, had been shown by Canet et al.[23] to possess long lifetimes that can be used to preserve magnetisation enhanced by a chemical reduction with *para*-hydrogen. The structure of acrylic acid, along with its 1D spectrum, is shown in Figure 1.

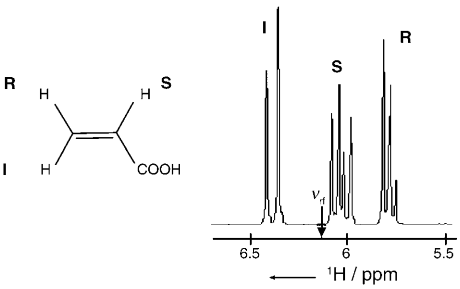

Figure 1. The molecular structure of acrylic acid and its 1D proton NMR spectrum at 500 MHz ($B_0 = 11.75$ T) and 298 K. The three protons *I*, *S*, and *R* are coupled by $J_{\mathrm{IS}} = 17.3$ Hz, $J_{\mathrm{IR}} = 1.4$ Hz, and $J_{\mathrm{SR}} = 10.4$ Hz[23,24,27]

For a 3-spin system, the diagonalisation of the Liouvillian yields 64 eigenstates.[19] The ones having the smallest real negative eigenvalues, and therefore the longest lifetimes, will be called quadratic terms *Q* and can be expressed as shown in Equation (2) as bilinear combinations of operators:

$$Q^{\mathrm{ISR}} = \frac{1}{\sqrt{3}}\left(\lambda_{\mathrm{IS}}\vec{I}\cdot\vec{S} + \lambda_{\mathrm{IR}}\vec{I}\cdot\vec{R} + \lambda_{\mathrm{SR}}\vec{S}\cdot\vec{R}\right) \tag{2}$$

A second type of long-lived states, which will be called cubic terms *C*, contain trilinear products of operators [Eq. (3)]:

$$\begin{gathered} C^{\mathrm{ISR}} = \mu_0 T_{\mathrm{x}} + \tfrac{\mu_{\mathrm{IS}}}{\sqrt{3}}\left[\left(\vec{I}\cdot\vec{S} - E/4\right)R_{\mathrm{x}} - T_{\mathrm{x}}\right] + \\ \tfrac{\mu_{IR}}{\sqrt{3}}\left[\left(\vec{I}\cdot\vec{R} - E/4\right)S_{\mathrm{x}} - T_{\mathrm{x}}\right] + \tfrac{\mu_{\mathrm{SR}}}{\sqrt{3}}\left[\left(\vec{S}\cdot\vec{R} - E/4\right)I_{\mathrm{x}} - T_{\mathrm{x}}\right] \end{gathered} \tag{3}$$

where *E* is the identity operator, and $T_{\mathrm{x}} = I_{\mathrm{x}}S_{\mathrm{x}}R_{\mathrm{x}}$ is a product of Cartesian spin operators that have a phase *x* parallel to the rf field $B_{1\mathrm{x}}$ that must be applied to sustain the long-lived state.

Under cw irradiation, the three protons effectively become magnetically equivalent, provided the *J*-couplings in the system are all equal. The symmetry-adapted eigenstates of the resulting A_3 system can be classified according to three irreducible representations (A, E an E′), much as for the three protons in methyl groups. Such eigenstates are best described in terms of tensor operators, as discussed elsewhere.[28]

The coefficients λ and μ in Equations (2) and (3) are set by the coherent part of the Hamiltonian and by the geometry of the system, which determines the fluctuating stochastic part of the Liouvillian that leads to relaxation. If we focus attention on non-oscillating eigenstates of the Liouvillian, we must fulfil the requirement of Equations (4) and (5) that the long-lived states commute with the *J*-coupling Hamiltonian:[22,23]

$$[O, H_{\mathrm{J}}] = 0 \tag{4}$$

with $O = \left\{Q^{\mathrm{ISR}}, C^{\mathrm{ISR}}\right\}$, and

$$H_{\mathrm{J}} = J_{\mathrm{IS}}\vec{I}\cdot\vec{S} + J_{\mathrm{IR}}\vec{I}\cdot\vec{R} + J_{\mathrm{SR}}\vec{S}\cdot\vec{R} \tag{5}$$

The requirement in Equation (4) leads to [Eq. (6)]:

$$\rho_{\mathrm{IS}}(J_{\mathrm{IR}} - J_{\mathrm{SR}}) + \rho_{SR}(J_{\mathrm{IS}} - J_{\mathrm{IR}}) = \rho_{IR}(J_{\mathrm{IS}} - J_{\mathrm{SR}}) \tag{6}$$

with $\rho \in \{\mu, \lambda\}$, since the commutators have the property $\left[\vec{I}\cdot\vec{R}, \vec{I}\cdot\vec{S}\right] = -\left[\vec{I}\cdot\vec{R}, \vec{S}\cdot\vec{R}\right]$

The property in Equation (4) ensures the stability of long-lived states towards the action of *J*-couplings.[9]

In the case of acrylic acid (Figure 1), the rotational correlation time $\tau_c \sim 22$ ps at 298 K could be inferred from the initial spin–lattice recovery rates of the protons $T_1(\mathrm{H}^{\mathrm{I}}) \sim T_1(\mathrm{H}^{\mathrm{R}}) = 5.7$ s and $T_1(\mathrm{H}^{\mathrm{S}}) = 22.5$ s (Figure 1). Considering this correlation time, the molecular structure, and the continuous-wave rf field with amplitude $\gamma B_{1x}/(2\pi) = 2.5$ kHz used to sustain the LLS, with the carrier set at the mean frequency of spins *I* and *R*, $\nu_0 = (\nu_{\mathrm{I}} + \nu_{\mathrm{R}})/2$ (Figure 1), the coefficients were found to be, for the quadratic state Q^{ISR} of Equation (2):

$$\lambda_{\mathrm{IS}} = -0.23,\ \lambda_{\mathrm{IR}} = 0.93,\ \text{and}\ \lambda_{\mathrm{SR}} = 0.28, \tag{7}$$

and for the cubic state C^{ISR} of Equation (3):

$$\mu_0 = 0.51,\ \mu_{\mathrm{IS}} = -0.19,\ \mu_{\mathrm{IR}} = 0.80,\ \text{and}\ \mu_{\mathrm{SR}} = 0.24. \tag{8}$$

Note that the squares of these coefficients sum up to 1, since the states are normalized. Neglecting the chemical shift anisotropies of the protons, the relaxation time constants for the quadratic and cubic states are predicted to be $T_{\mathrm{LLS}}(Q^{\mathrm{ISR}}) = 37$ s and $T_{\mathrm{LLS}}(C^{\mathrm{ISR}}) = 50$ s. These can be compared with the longest predicted lifetime of Zeeman polarization $T_1(\mathrm{H}^{\mathrm{S}}) = 22.5$ s, so that the ratios are $T_{\mathrm{LLS}}(Q^{\mathrm{ISR}})/T_1(\mathrm{H}^{\mathrm{S}}) = 1.6$ and $T_{\mathrm{LLS}}(C^{\mathrm{ISR}})/T_1(\mathrm{H}^{\mathrm{S}}) = 2.2$. However, if one introduces non-vanishing anisotropies of the chemical shifts (which requires making some unwarranted assumptions about the tensors), the lifetime of C^{ISR} decreases faster than that of Q^{ISR}, so that the latter quadratic state is likely to be the longest-lived state under most conditions, except in very low fields.

Once a state has been identified with a relaxation time constant TLLS that is sufficiently long to study slow dynamic processes, a pulse sequence may be optimised in silico, in order to prepare this state (Figure 2). Since states described by the

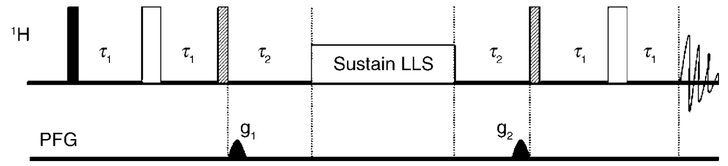

Figure 2. Pulse sequence allowing for optimum excitation of long-lived states in three-spin systems. Hatched, black, and open rectangles stand for (π/4), (π/2), and π pulses, respectively. All pulses are along the *x*-axis, except for the (π/4) pulses, applied along the *y*-axis.

scalar products $\vec{I}\cdot\vec{S}$, $\vec{I}\cdot\vec{R}$, and $\vec{S}\cdot\vec{R}$ are constituents of the quadratic states Q^{ISR}, the starting point must be a sequence designed to excite terms of the type $\vec{I}\cdot\vec{S}$, $\vec{I}\cdot\vec{R}$, and $\vec{S}\cdot\vec{R}$. Numerical optimization allows one to obtain coefficients λ_{IS}, λ_{IR}, and λ_{SR} so as to fulfil the commutator of Equation (4). Pulse sequences with the structure shown in Figure 2 were optimised to excite Q^{ISR} operators in the molecules listed in Table 1. These states can be sustained by continuous-wave irradiation prior to re-conversion into detectable coherences. The numerical calculations were performed using the GAMMA libraries[29] as previously described,[17,30,31] considering a continuous-wave rf field with an amplitude $\gamma B_{1x}/(2\pi) = 2.5$ kHz.

Table 1. Experimental time constants T_1 and T_{LLS} of protons at 500 MHz ($B_0 = 11.75$ T) and 298 K. Paramagnetic oxygen content was reduced by bubbling gaseous nitrogen through the samples.

Molecule	$T_1(H^I,H^R)$ (s)	$T_1^{max} = T_1(H^S)$ (s)	T_{LLS} (Q^{ISR}) (s)	T_{LLS}/T_1^{max}
Acrylic Acid	5.733 ± 0.003	22.472 ± 0.004	52.5 ± 0.2	2.3
Aspartic Acid	1.237 ± 0.003	5.842 ± 0.004	10.9 ± 0.2	1.9
Asparagine	1.208 ± 0.003	5.353 ± 0.004	11.2 ± 0.1	2.1
Serine	1.753 ± 0.003	7.592 ± 0.004	13.55 ± 0.04	1.8
Cysteine	1.717 ± 0.003	7.682 ± 0.004	12.42 ± 0.08	1.6
Histidine	0.984 ± 0.001	4.901 ± 0.003	5.3 ± 0.1	1.1

In the amino-acids cysteine, serine, aspartic acid, asparagine, and histidine, the three-spin proton systems were analysed. For aspartic acid, by way of example, the coefficients λ in the quadratic term Q^{ISR} of Equation (2) are:

$$\lambda_{IS} = -0.02,\ \lambda_{SR} = -0.07,\ \text{and}\ \lambda_{IR} = -0.99 \qquad (9)$$

The delays τ_1 and τ_2 were optimized numerically to obtain a maximum coefficient of the state Q^{ISR}. The best results were found for $\tau_1 = 14$ ms and $\tau_2 = 15.5$ ms. Using these values, the coefficient of the desired state at the beginning of the "*sustain LLS*" period was $<Q^{ISR}> = 0.9$. Unwanted coherences are destroyed by gradients.

For systems featuring two nearby non-equivalent spins *I* and *R*, such as the $H^{\beta 1}$ and $H^{\beta 2}$ protons in the side-chains of the amino acids mentioned above and a remote spin *S*, for example, the H^{α} proton in these amino acids, the conventional longitudinal relaxation time constant $T_1^{max} = T_1(H^S)$ is longer than $T_1(H^R) \approx T_1(H^I)$. In Table 1, the longest relaxation time constants in the molecule, fitted from initial recovery for spins H^S for different amino acids, are compared to the $T_{LLS}(Q^{ISR})$ of Equation (2) that could be excited. The gain in lifetimes afforded by the use of LLS, as quantified by the T_{LLS}/T_1^{max} ratio, is approximately a factor two for all systems except for histidine, where dipolar interactions with nearby ring protons significantly reduce T_{LLS}. The slow decay of the LLS in aspartic acid is compared in Figure 3 with the decay of the deviation from equilibrium of the longitudinal magnetisation of spin H^S.

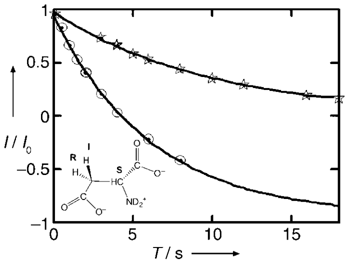

Figure 3. Fits of the exponential recovery with the longest time-constant in aspartic acid, $T_1^{max} = 5.842 \pm 0.004$ s, and of the decay of the long-lived state, $T_{LLS} = 10.9 \pm 0.2$ s.

We have explored the existence of LLS in taurine and glycerol dissolved in D_2O (Figure 4). In taurine, the four methylene protons constitute an A_2X_2 system with a spectrum consisting of two triplets.[32] Long-lived states containing scalar products $\vec{I}\cdot\vec{S}$, with $I = A$ and $S = X$, have been found to have lifetimes $T_{LLS} = 2.7 \pm 0.1$ s that are longer than $T_1^{max} = 1.02 \pm 0.03$ s at 800 MHz and 298 K. The survival of these long-lived states is ensured by the fact that the generalised state is a sum of scalar products with equal weights, which commutes with the *J*-coupling Hamiltonian. In glycerol, the five protons constitute an AA′BB′M system featuring long-lived states containing scalar products, $\vec{I}\cdot\vec{S}$, $\vec{I}\cdot\vec{R}$, and $\vec{S}\cdot\vec{R}$, with $I = H^A$, $S = H^M$, and $R = H^B$. Nearly exponential decays with $T_{LLS} = 7.34 \pm 0.03$ s were obtained, while the maximum spin–lattice relaxation time constant was $T_1^{max} = T_1(H^M) = 3.585 \pm 0.004$ s at 500 MHz and 298 K.

Long-lived spin states that are apt to store magnetisation over periods significantly longer than the longitudinal spin–lat-

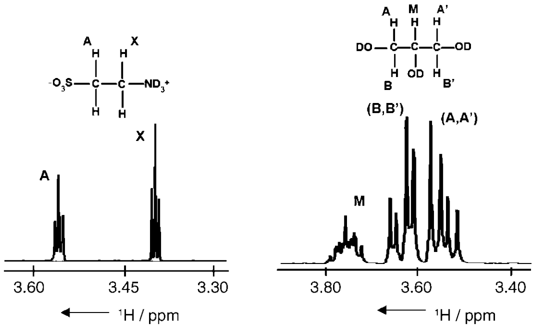

Figure 4. Molecular formulae and 1D spectra measured at 400 MHz and 298 K for taurine (left) and glycerol (right) dissolved in D_2O.

tice relaxation times can be obtained in selected amino acids and other small molecules of biological interest. Provided LLS are isolated from spins with high gyromagnetic ratios in their close environment, it will be interesting to investigate whether these methods allow for long lifetimes to be obtained even in molecules with high molecular mass, since the dipolar interaction within the group of spins involved in the LLS is silenced independently of the molecular correlation time. It has been observed that a partial deuteration of the immediate environment of the two coupled spins can lead to a significant improvement in the lifetimes, as an extension of the singlet-state lifetime by more than one order of magnitude was obtained for protons 5′ and 5′′ in a saccharide[14]. It is now possible to label biomolecules leaving only a few selected proton pairs in a fully deuterated background. For example, proteins that only contain aromatic protons in Phe, Tyr and Trp can be prepared by adding the aromatic amino acid precursor shikimic acid to fully deuterated media.[33] Singlet states may then be excited on the ring protons of tyrosines, for instance. The identification of LLS in spin systems with more than two spins significantly alleviates the task of deuteration strategies required to isolate the LLS from the environment. Though partial deuteration may be necessary, we show herein that a gain in spin memory of a factor two may be obtained using LLS without recurring to nitrogen-15 or carbon-13 labelling. The structure of these states is discussed to show how they can be excited and sustained. This is of interest for studies of diffusion, where an extension of the lifetimes allows one to reduce the strength of the gradients,[16,31,34] and to study anomalous long-term behaviour of restrained diffusion in porous materials. To study chemical exchange, the lifetimes of magnetisation have to match the timescale of the process.

Experimental Section

The experiments were carried out at 500 MHz ($B_0 = 11.75$ T) and 298 K using ~20 mM solutions in deuterated water. Gaseous nitrogen was bubbled through the samples during five minutes prior to the experiments in order to reduce the content of dissolved oxygen. Eight transients were acquired for each experiment using a relaxation delay of 30 s. Experimental relaxation time constants T_1^{max} and T_{LLS} were obtained by fitting normalized peak intensities to mono-exponentially decaying functions. Continuous-wave irradiation with an amplitude $\nu_1 = 2.5$ kHz was used to sustain the LLS.

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