Measurement of Relaxation Rates in Crowded NMR Spectra by Selective Coherence Transfer

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Abstract: In crowded NMR spectra, it is shown how longitudinal relaxation rates (laboratory-frame rates $1/T_1$) can be measured by selective inversion–recovery of a chosen site A followed by selective transfer of magnetization to another site X through a scalar coupling J_{AX}, prior to observation of the signal. Relaxation rates in the presence of spin-locking (rotating frame rates $1/T_{1\rho}$) can be measured by first transferring transverse magnetization selectively from site X to A, followed by selective spin-locking of the magnetization of A, which can then be observed after partial decay. In both cases, the transfer can be achieved by a doubly-selective homonuclear Hartmann–Hahn method that uses simultaneous spin-locking of the transverse magnetization components of sites A and X by sidebands of an audio-modulated radio-frequency field. Provided the A–X cross-peak multiplet in a two-dimensional correlation ("COSY") spectrum does not suffer from overlap, there is no ambiguity in the one-dimensional spectra resulting from the novel methods. The techniques make it possible to measure accurate self-relaxation rates ρ or ρ^t (diagonal elements of the Solomon matrices), which are important for a quantitative analysis of Overhauser effects in either laboratory or rotating frames. The methods are applied to the protein bovine pancreatic trypsin inhibitor (BPTI) and to the cyclic undecapeptide cyclosporin A (CsA).

Introduction

Although the measurement of longitudinal and transverse relaxation rates of protons has now become a matter of routine in small and medium-sized molecules,[1,2] this remains difficult if there are extensively overlapping resonances, as in proton spectra of proteins, nucleic acids, and other macromolecules. Since the advent of two-dimensional spectroscopy, there has been an increasing tendency to rely primarily on the measurement and analysis of *cross*-relaxation rates (σ_{AX} and σ^t_{AX} in the laboratory and rotating frames), which can be readily obtained with nuclear Overhauser effect spectroscopy (NOESY),[3] its rotating frame equivalent (ROESY),[4] and related techniques.[5] Unfortunately, there has been a concomitant tendency to neglect the measurement of *self*-relaxation rates ρ_A and ρ^t_A, which are much more difficult to determine if the spectra feature heavily overlapping resonances. It is possible to combine the idea of inversion–recovery with the resolving power of two-dimensional spectroscopy,[6-8] but this approach does not appear to enjoy widespread popularity, perhaps because it demands a great deal of spectrometer time. Yet it is obvious that measurements of self-relaxation rates would be of utmost practical importance. With reference to Solomon's relaxation matrix,[5,9] cross-relaxation rates σ_{AX} or σ^t_{AX} correspond to off-diagonal elements, while self-relaxation rates ρ_A or ρ^t_A correspond to diagonal elements. It is obvious that the analysis of Overhauser effects is fraught with dangers if we merely measure cross-relaxation rates. In particular, during the exploration of our so-called "synchronous nutation" method,[10] which is intended to measure cross-relaxation rates between selected pairs of spins, it became apparent that there is an urgent need for developing new methods for measuring self-relaxation rates.

The first of the schemes that we propose is shown in Figure 1. Prior to setting up the experiment, one must identify a spin X that has a scalar coupling J_{AX} to the spin A under investigation. Such a partner can be found by inspection of a COSY spectrum. Initially, a selective inversion pulse, such as a G^3 Gaussian cascade,[11] is applied at the chemical shift Ω_A of the spin of interest to invert its magnetization selectively ($I_z^A \rightarrow -I_z^A$). After a relaxation interval τ_{REL}, which may be incremented from one experiment to the next, a selective Gaussian-shaped pulse with a 270° on-resonance flip angle (a so-called self-refocusing G^1 pulse[12]) is applied, again at the frequency Ω_A, to convert the (partly recovered) longitudinal component of spin A into transverse magnetization ($-I_z^A \rightarrow I_x^A$). This is followed by a doubly-selective irradiation period of duration $\tau_{DSI} \approx 1/J_{AX}$, which leads to a transfer $I_x^A \rightarrow I_x^X$ through a homonuclear Hartmann–Hahn effect.[13] During the period τ_{DSI}, the radio-frequency transmitter is positioned halfway between the two chemical shifts, $\omega_{rf} = {}^1/_2(\Omega_A + \Omega_X)$, and the rf amplitude is modulated with $\omega_a = {}^1/_2(\Omega_A - \Omega_X)$, so that the resulting sidebands coincide with the two chemical shifts. It is not crucial to the success of the experiment that the shifts and coupling constant be known very accurately. In particular, if τ_{DSI} is misset ($\neq 1/J_{AX}$), the transfer will be less than complete; as a result, the signals will be attenuated uniformly for all values of τ_{REL}, but this does not affect the evaluation of the recovery curve.

Figure 2 shows an inversion–recovery measurement obtained in this manner. The spectra of bovine pancreatic trypsin inhibitor (BPTI) dissolved in ^2H_2O at 327 K were obtained with a Bruker MSL 300 spectrometer equipped with a selective excitation unit. We focus attention on the aromatic protons H^ϵ and H^δ of the Tyr^{23} residue. Because of rapid rotation, the shifts of H^ϵ and $H^{\epsilon'}$ at 6.30 ppm and of H^δ and $H^{\delta'}$ at 7.20 ppm are pairwise degenerate.[14] The multiplet of H^ϵ can be observed directly, but the signal of H^δ is buried under other resonances, as may be appreciated from the aromatic region shown in the inset. The lower part of Figure 2 shows a conventional inversion–recovery measurement of H^ϵ. We used selective pulses for both inversion and monitoring but skipped the doubly-selective irradiation ($\tau_{DSI} = 0$ in the sequence of Figure 1). The recovery is essentially exponential with a self-relaxation rate $\rho(H^\epsilon) = 2.26\ s^{-1}$. The upper part of Figure 2 shows the inversion–recovery behavior of H^δ, *observed indirectly* with the sequence of Figure 1 by transferring magnetization from H^δ to H^ϵ via the scalar coupling $J(H^\delta,H^\epsilon) \approx 9$ Hz with $\tau_{DSI} = 111$

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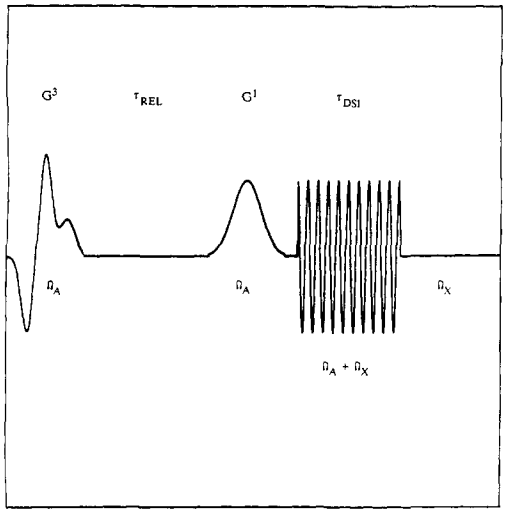

Figure 1. Pulse sequence starting with selective inversion of spin A by a G^3 Gaussian cascade applied at the chemical shift Ω_A, followed by a relaxation delay τ_{REL} to allow for partial recovery, by a 270° Gaussian pulse G^1 at Ω_A and by doubly-selective irradiation at $\Omega_A + \Omega_X$ for a duration $\tau_{DSI} \approx 1/J_{AX}$ to transfer magnetization from A to X.

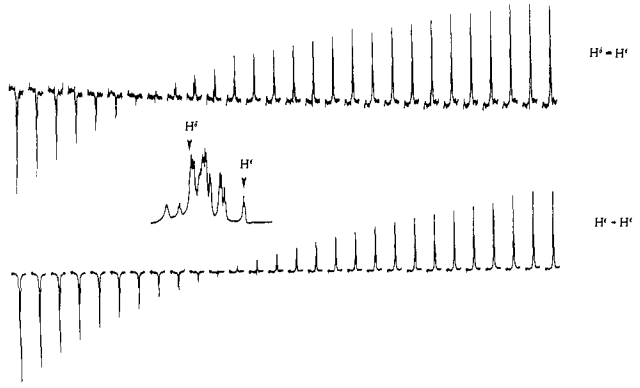

Figure 2. Selective inversion–recovery measurements of the aromatic protons H^ϵ and H^δ of the Tyr^{23} residue in bovine pancreatic trypsin inhibitor (BPTI) dissolved in ^2H_2O at 327 K. The inset shows the aromatic region (between 8.2 and 5.8 ppm) of the one-dimensional spectrum (eight scans); the arrows indicate the shifts of H^ϵ at 6.30 ppm and of H^δ at 7.20 ppm. Lower part: normal inversion–recovery measurement of H^ϵ in Tyr^{23} using selective pulses and direct observation of H^ϵ; for each multiplet, 16 scans were acquired; the complete series of 28 experiments with τ_{REL} incremented between 0 and 810 ms in steps of 30 ms required only 8 min. Upper part: inversion–recovery of H^δ using selective pulses and Hartmann–Hahn transfer from H^δ to H^ϵ with the sequence of Figure 1, using τ_{DSI} = 111 ms, γB_1 = 50 Hz for each sideband (100 Hz total), a G^3 cascade of 30-ms duration with an rf amplitude adjusted for vanishing transverse magnetization on-resonance, and a 270° G^1 pulse of 30 ms calibrated for maximum transverse magnetization. For each multiplet, 64 scans were acquired; the series of 28 experiments required about 1 h.

ms. Direct observation is not possible in this case because of overlap. The recovery is again roughly exponential, with a rate $\rho(H^\delta) = 3.76\ s^{-1}$. The difference of the two self-relaxation rates $\rho(H^\delta) - \rho(H^\epsilon) = 1.50\ s^{-1}$ must be attributed to the fact that H^δ is exposed to fluctuating dipolar fields originating in the neighboring H^β and $H^{\beta'}$ protons, while H^ϵ is more isolated. If one wishes to analyze the cross-relaxation behavior of these protons in a quantitative manner, it is essential that such differences in self-relaxation rates be correctly measured.[10]

Figure 3 shows the inversion–recovery behavior of the H^β and $H^{\beta'}$ protons of the same Tyr^{23} residue in BPTI, which resonate at 2.71 and 3.49 ppm, respectively,[14] and which are both largely hidden under overlapping resonances (see inset). In these cases, the relaxation behavior may be observed indirectly by transferring magnetization via the scalar coupling $|J(H^\beta,H^{\beta'})| \approx 12$ Hz, so that we observe the recovery of H^β by transferring the signal to $H^{\beta'}$,

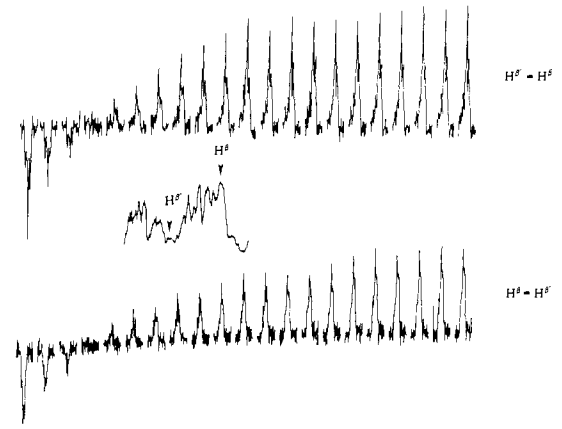

Figure 3. Inversion–recovery measurements of the H^β and $H^{\beta'}$ protons of Tyr^{23} in BPTI (conditions as in Figure 2, except that τ_{DSI} = 80 ms). The inset shows the crowded β region; arrows indicate the shifts of H^β at 2.71 ppm and of $H^{\beta'}$ at 3.49 ppm. Bottom: inversion–recovery of H^β with transfer to $H^{\beta'}$. Top: inversion–recovery of $H^{\beta'}$ with transfer to H^β. For each multiplet, 256 scans were acquired; each series of experiments with τ_{REL} incremented from 0 to 600 ms in steps of 30 ms required 3 h.

and vice versa. In these cases, the decay curves are apparently not exponential, but the initial rates yield $\rho(H^\beta) = 7.17\ s^{-1}$ and $\rho(H^{\beta'}) = 5.95\ s^{-1}$. The difference could be explained if H^β were closer to H^α than $H^{\beta'}$, so that H^β would experience stronger fluctuating fields. Although this interpretation must be regarded as tentative at this stage, the measurements demonstrate that our method can be applied successfully even when *both* coupled partners A and X are buried in crowded regions of the spectrum. There is no ambiguity in the transfer, provided the A–X cross-peak multiplet in a two-dimensional COSY spectrum does not suffer from overlap. If by accident such an overlap occurs, one may combine several consecutive transfer steps A → M, M → X, etc., so that one can in effect have the resolving power of three- and higher-dimensional spectroscopy.[13]

Note that in crowded spectra the initial selective inversion pulse applied at Ω_A will actually affect several protons that are accidentally degenerate in shift with spin A. Except in specific circumstances (e.g., when two diastereotopic protons are nearly degenerate), we may safely assume that the other protons that are inverted unwittingly are spatially remote from A (say, by more than 500 pm), so that their inversion cannot affect the recovery behavior of A.

To estimate cross-relaxation rates (Overhauser effects) in the presence of a spin-locking field, one may use the ROESY method[4] to measure σ^l_{AX}. For a quantitative analysis, it is necessary to supplement this information by measuring the self-relaxation rates ρ^l_A and ρ^l_X, which correspond to diagonal elements of the Solomon matrix that describes relaxation in the rotating frame. These rates are often referred to as "rotating frame relaxation rates" $1/T_{1\rho}{}^A$ and $1/T_{1\rho}{}^X$. To measure these rates, we propose the experiment shown in Figure 4, which uses *selective spin-locking* of the magnetization of interest. Prior to setting up the experiment, one must again identify a spin X that has a scalar coupling J_{AX} to the spin A under investigation. A selective 270° Gaussian-shaped G^1 pulse[12] is applied at Ω_X, immediately followed by a doubly-selective irradiation period of duration $\tau_{DSI} \approx 1/J_{AX}$, which leads to a Hartmann–Hahn transfer[13] $I_x{}^X \rightarrow I_x{}^A$. A selective, monochromatic spin-locking pulse of (variable) duration τ_{REL} is applied at Ω_A, and the signal of the (partly relaxed) spin A is then observed directly. The phase of the first pulse must be alternated together with the receiver phase. If overlap is severe, another Hartmann–Hahn transfer may be envisaged, preferably to another coupling partner M.

Figure 5 shows an application of the method of Figure 4 to a sample of the cyclic undecapeptide cyclosporin A (CsA) dissolved in $CDCl_3$ at 303 K. The magnetization was initially transferred by doubly-selective irradiation of duration τ_{DSI} = 100 ms from the H^α proton of *N*-methylleucine-9, which resonates at 5.7 ppm, to the H^β proton at 2.13 ppm,[15] through the coupling $J(H^\alpha,H^\beta)$

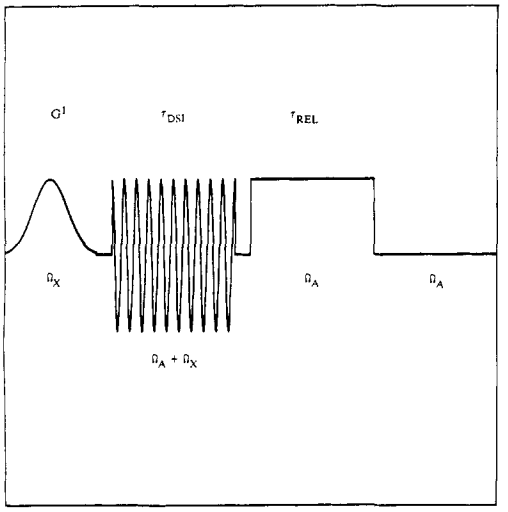

Figure 4. Pulse sequence for measurement of relaxation of spin A during selective spin-locking ($T_{1\rho}$). Initially, transverse magnetization of spin X is excited by a 270° Gaussian pulse G^1 at Ω_X, and a doubly-selective irradiation at $\Omega_A + \Omega_X$ with a duration $\tau_{DSI} \approx 1/J_{AX}$ leads to a transfer from X to A through a homonuclear Hartmann–Hahn effect. The resulting A magnetization is then spin-locked by selective monochromatic irradiation at Ω_A for a variable duration τ_{REL}, and the signal of A is finally detected.

= 11.2 Hz.[16] The decay in Figure 5 shows the $T_{1\rho}$ relaxation of the multiplet of proton H^β, which appears to be roughly exponential with $1/T_{1\rho} = \rho^t(H^\beta) \approx 5.55\ s^{-1}$.

In the example of Figure 5, all multiplet components appear to have a similar behavior, indicating that cross-correlation is negligible in this case.[17] Bull has recently suggested[18] that selective spin-locking may provide an elegant means to probe cross-correlation effects. We have shown recently, both by theory and experiment,[19] that selective spin-locking makes it possible to study not only cross-correlation of fluctuations of pairs of dipolar couplings but also cross-correlation of the fluctuations of an anisotropic chemical shift and a dipolar interaction. The method of Figure 4 allows one to extend such studies to crowded spectra.

The idea of preparing a suitably tailored initial state by a selective Hartmann–Hahn transfer is reminiscent of the idea of "injection" of coherence,[20] where magnetization is transferred

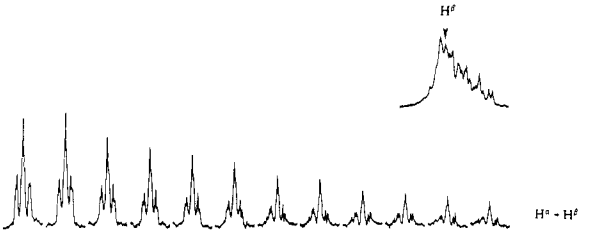

Figure 5. Decay during selective spin-locking of the H^β proton of *N*-methylleucine-9 in cyclosporin A dissolved in $CDCl_3$ at 303 K. The inset shows the crowded region in the one-dimensional spectrum, the arrow indicating the chemical shift of H^β at 2.13 ppm. Coherence was first transferred selectively from the H^α proton of the same residue at 5.70 ppm with a doubly-selective irradiation period τ_{DSI} = 90 ms with γB_1 = 40 Hz for each sideband (80 Hz total), after a 270° G^1 pulse of 30 ms calibrated for maximum transverse magnetization and followed by a single-frequency spin-locking interval with γB_1 = 40 Hz. The duration of this interval was incremented from 0 to 220 ms in steps of 20 ms. The number of scans was 16, and the complete series of experiments required 6 min.

through selective pulses in order to separate overlapping multiplets.

In principle, the rates reported here could also be determined by combining nonselective inversion–recovery or nonselective spin-locking with a COSY sequence to map out the amplitudes of cross-peaks as a function of the relaxation delay,[6-8] but the duration of the experiments would tend to be prohibitive. By contrast, the acquisition of each of the signals in the upper part of Figure 2 required only 2 min, those in Figure 3 about 9 min, and those in Figure 5 about 1 min. This makes it possible to use small increments of τ_{REL}, so that one can appreciate to what extent the recovery is mono- or multiexponential.

Doubly-selective methods obviously demand that a different experiment be set up for each proton of interest. This does not seem unreasonable, since most studies of molecular mobility in biological macromolecules are concerned with some particular region of the molecule, for example, some part of the rigid backbone, some mobile side chains, or some putative contact site. Thus we feel that the selective character of our approach befits the type of questions that are likely to arise in relaxation studies of macromolecules.

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