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# Apparent Transverse Relaxation Rates in Systems with Scalar-Coupled Protons

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Spin echo experiments[1−3] are extensively used to determine homogeneous transverse relaxation rates when they are masked by inhomogeneous broadening,[4] to evaluate translational diffusion coefficients[5−7] and to separate line width contributions arising from chemical exchange and homogeneous transverse relaxation.[8−13] The modulation of echoes by homonuclear scalar couplings may render the determination of transverse relaxation rates of individual spins difficult, in particular for molecules that are isotopically enriched in $^{13}C$ or $^{15}N$, and of course for scalar-coupled protons. To avoid echo modulations, most studies using refocusing pulses have so far been restricted to isolated or selectively labeled $^{13}C$ or $^{15}N$ spins. Herein we demonstrate measurements of apparent $^{1}H$ transverse relaxation rates of backbone and side-chain protons in Cyclosporin A (CsA) determined by quenching echo modulations that arise from homonuclear scalar couplings ($^{m}J$ with $m \geq 2$). Because of the cumulative effects of nonideal pulses with 'tilted' effective fields, modulations in Carr−Purcell−Meiboom−Gill (CPMG) multiple-refocusing sequences $\pi/2 - [\tau - \pi - \tau]_{2n}$ can be quenched for $\nu_{rep} \neq \Omega_S/(2k\pi)$, where $k$ is an integer,[14] using refocusing pulses of moderate strength with the radio frequency (*rf*) carrier on-resonance for a spin $I$ ($\Omega_I = 0$) under investigation and pulse repetition rates $\nu_{rep} = 1/(2\tau + \tau_\pi)$ that are smaller than the offset $\Omega_S$ of the main coupling partner $S$. Selective refocusing pulses do not offer an attractive alternative, for they would have to be quite long, so that relaxation and evolution under the scalar couplings during the pulses would have to be taken into account.

CsA is a cyclic undecapeptide of great pharmaceutical importance.[15] The NMR spectra have been assigned by Oschkinat et al.[16] The chemical structure and the numbering of the amino acids is shown in Figure 1A. A TLC grade sample was obtained from Sigma Aldrich and dissolved in $CDCl_3$. The 1D $^{1}H$ spectrum displayed in Figure 1B shows many well-resolved multiplets in the $H^N$ and $H^\alpha$ regions and a few nonoverlapping methyl peaks. In order to choose the most favorable *rf* amplitudes and repetition rates, the offsets of the coupling partners were determined from a COSY spectrum. The integrals of a dozen selected multiplets (Supporting Information) were recorded using the so-called 'hybrid' approach,[14] *i.e.*, by varying the delay $\tau$ while keeping $n$ constant (Figure 2A). In this manner, favorable quenching conditions can easily be identified empirically without resorting to theory or simulations.

The peaks marked '*a*' to '*l*' in Figure 1B have been investigated by recording unmodulated echo decays, after choosing favorable $\tau$ delays and incrementing $n$. For the methyl protons $H^\gamma$ in Val-5 (multiplet *a*), the offset to the neighboring $H^\beta$ protons was only 710 Hz while $^{3}J(H^\beta H^\gamma) = 6.55$ Hz and the *rf* amplitude was 5.6

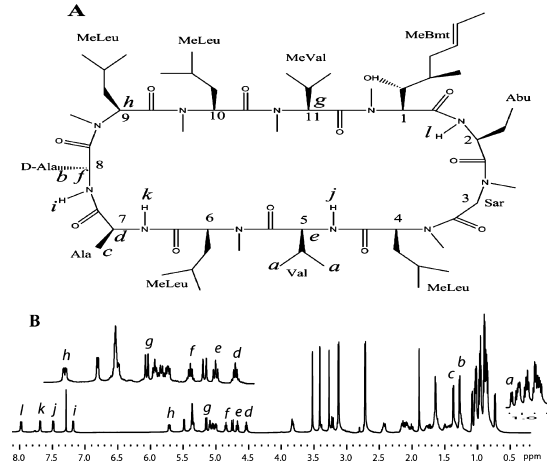


***Figure 1.*** (A) Chemical structure of CsA with numbering of amino acids 1 to 11 and labeling of a few selected protons *a* to *l*. (B) Proton spectrum of CsA in $CDCl_3$ at 500 MHz and 300 K. The three methyl doublets labeled '*a*', '*b*', and '*c*' at 1.08, 1.27, and 1.37 ppm stem from Val-5, D-Ala-8, and Ala-7; five $H^\alpha$ multiplets labeled '*d*', '*e*', '*f*', '*g*', and '*h*' at 4.53, 4.67, 4.84, 5.15, and 5.72 ppm are due to Ala-7, Val-5, D-Ala-8, MeVal-11, and MeLeu-9; four amide $H^N$ signals labeled '*i*', '*j*', '*k*', and '*l*' at 7.18, 7.48, 7.68, and 7.98 ppm are identified with D-Ala-8, Val-5, Ala-7, and Abu-2.

kHz. The $R_2^{app}$ rates were determined by exponential fitting. By way of example, consider the doublet of the proton $I = H^\alpha$ of MeVal-11 (signal '*g*' in Figure 1B). The *rf* carrier was positioned at 5.15 ppm to be resonant with this $H^\alpha$ proton. Since there is only one resolved coupling $^{3}J(H^\alpha H^\beta) = 10.9$ Hz, the system can be treated as a two-spin system, provided one limits the observation to the $H^\alpha$ region. The offset of the coupling partner $H^\beta$ is $\Omega_S/(2\pi) = 1.5$ kHz. The $^{4}J$ couplings to the six protons of the two $C^\gamma H_3$ groups and the three protons of the $NCH_3$ group are not resolved. Figure 2A displays the amplitude of the integral of the multiplet obtained by Fourier transformation of the 60th echo ($n = 30$), as a function of $\tau$, i.e., using the hybrid approach. For the 500th echo ($n = 250$), the $^{4}J$ couplings to the nine remote protons give rise to three other weak 'dips' for $\tau = 430$, 677, and 705 $\mu$s (not shown).

Figure 2B shows experimental decays of the $H^\alpha$ proton '*g*' recorded for increasing $n$, using $\tau$ intervals chosen to avoid echo modulations. All curves appear to be free of echo modulations, and $R_2^{app}$ can be determined from simple monoexponential fits. These rates are compared with experiments where the decay of the echoes was monitored with a single refocusing $\pi$ pulse of duration $\tau_\pi$ applied at $T/2 = n(2\tau + \tau_\pi) - \tau_\pi/2$ (Figure 2C). Fitting with a monoexponential decay multiplied by a cosine function (i.e., assuming a two-spin system) gives higher decay rates. It is difficult to define a fitting function that takes into account all unresolved $^{4}J$

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***Table 1.*** Apparent Relaxation Rates $R_2^{app}$ Determined by Single and Multiple Refocusing Experiments for a Few Selected Protons in CsA (Figure 2 Gives Details for the $H^{\alpha}$ Proton '*g*' of MeVal-11)

| Label in Figure 1 (ppm) | Residue and proton type | Couplings to neighbors | Main $^3J$ (Hz) | Offset of main coupling partner (Hz) | Ratio $\Omega/\omega_1$ | Delays $\tau$ ($\mu$s) where echo modulations are worst | Best delays $\tau$ ($\mu$s) for quenching ($\nu_{rep}$, Hz) | Even numbered echoes 2*n* observed | $R_2^{app}$ ($s^{-1}$) from multiple refocusing | $R_2^{app}$ ($s^{-1}$) from single refocusing |
|---|---|---|---|---|---|---|---|---|---|---|
| *d*(4.53) | Ala-7 | $^3J(H^{\alpha}H^{\beta})$ | 7.2 | 1581 | 0.28 | 295 | 700 (672) | 1,6,..180 | 1.23 | 6.89 |
| | $CH^{\alpha}$ | $^3J(H^{\alpha}H^{N})$ | 7.4 | 1576 | 0.28 | 556 | 750 (629) | 1,6,..180 | 1.24 | 6.65 |
| | | $^4J(H^{\alpha}H^{N}_{i+1})$ | | | | 929 | 800 (592) | 1,6,..180 | 1.22 | 6.71 |
| *f*(4.84) | D-Ala-8 | $^3J(H^{\alpha}H^{\beta})$ | 8 | 1168 | 0.21 | 258, 494 | 350 (1267) | 1,10,..300 | 1.89 | N/A |
| | $CH^{\alpha}$ | $^3J(H^{\alpha}H^{N})$ | 6.75 | 1785 | 0.32 | 819, 405 | 580 (801) | 1,6,..180 | 1.78 | N/A |

couplings to the nine remote protons of the three methyl groups. For larger biomolecules with faster relaxation and broader lines, fitting both $R_2^{app}$ and $J$ will be even more difficult.

When the main coupling $^3J(H^{\alpha}H^{\beta})$ is quenched, long-range couplings have very weak effects. These can be quenched by using sequences with $750 < \tau < 850$ $\mu$s. Table 1 shows two examples for protons *d* and *f*. Ten other examples are illustrated in the Supporting Information.

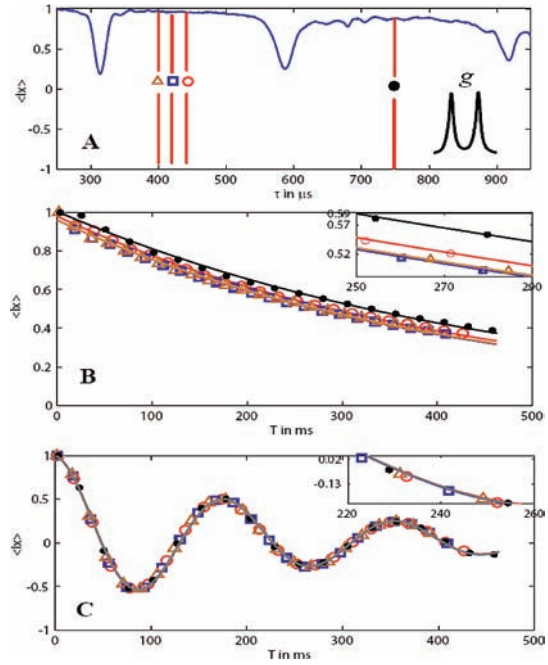


***Figure 2.*** (A) Amplitude of the $H^{\alpha}$ proton doublet '*g*' of MeVal-11 in CsA recorded with the hybrid sequence $[\tau - \pi_x - \tau]_{2n}$ as a function of $\tau$ at the top of the 60th echo ($n = 30$). The doublet arises from $^3J(H^{\alpha}H^{\beta}) = 10.9$ Hz. The *rf* carrier is positioned on resonance at 5.15 ppm, and the offset of the coupling partner $H^{\beta}$ is $\Omega_S/(2\pi) = 1.5$ kHz. The *rf* amplitude of the refocusing pulses is $\omega_1/(2\pi) = 5.605$ kHz (pulse length $\tau_\pi = 89.2$ $\mu$s), i.e., $\Omega_S/\omega_1 = 0.27$. The favorable intervals $\tau = 400$, 420, 440, and 750 $\mu$s are marked with triangles, squares, and open and filled circles. (B) Decays of the $H^{\alpha}$ proton doublet '*g*' of MeVal-11 recorded with the sequence $[\tau - \pi_x - \tau]_{2n}$ for these favorable $\tau$ intervals as a function of the number of cycles *n*. The unmodulated decays were fitted with monoexponential functions. Open circles: $\tau = 440$ $\mu$s ($\nu_{rep} = 1032$ Hz) and $n = 1$, 10, 20, ..., 220, so that the time axis $T = n(4\tau + 2\tau_\pi)$ extends over $0 < T < 426$ ms, and $R_2^{app} = 2.32$ $s^{-1}$. Open squares: $\tau = 420$ $\mu$s ($\nu_{rep} = 1076$ Hz), $0 < T < 409$ ms, and $R_2^{app} = 2.39$ $s^{-1}$. Triangles: $\tau = 400$ $\mu$s ($\nu_{rep} = 1125$ Hz), $0 < T < 391$ ms, and $R_2^{app} = 2.37$ $s^{-1}$. Filled circles: $\tau = 750$ $\mu$s ($\nu_{rep} = 629$ Hz), $n = 1$, 8, 16, ..., 144, $0 < T < 458$ ms, and $R_2^{app} = 2.13$ $s^{-1}$. (C) Decay of the $H^{\alpha}$ proton doublet '*g*' obtained with a single refocusing $\pi$ pulse applied at *T*/2. A fit with an exponential function multiplied by a cosine function gives an average value of $R_2^{app} = 3.93$ $s^{-1}$ and $J = 10.9$ Hz.

For the $H^{\alpha}$ protons '*d*' of Ala-7 and '*f*' of D-Ala-8, we notice that $R_2^{app}$('*d*') = 1.22 $s^{-1}$ < $R_2^{app}$('*f*') = 1.78 $s^{-1}$. This trend is consistent with the longitudinal relaxation rates $R_1$('*d*') = 0.95 $s^{-1}$ < $R_1$('*f*') = 1.31 $s^{-1}$. The $H^{\alpha}$ proton '*f*' of D-Ala-8 is in a crowded environment, as can be seen from a 3D space-filling model of CsA and from the strong NOESY cross peak between $H^{\alpha}$ of D-Ala-8 and the N-methyl protons of MeLeu-9.[15a] Generally, we found that $R_2^{app}(-NH) > R_2^{app}(-CH_3) > R_2^{app}(-CH)$ for the 12 protons that we investigated in detail.

In conclusion, our experiments demonstrate that relaxation rates of backbone and side-chain protons in peptides can be determined by quenching homonuclear scalar couplings. The measurement of apparent proton transverse relaxation rates opens new avenues for dynamic studies.

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**Supporting Information Available:** Table S1 with 10 further examples of resonances with their apparent relaxation rates in CsA. This material is available free of charge via the Internet at http://pubs.acs.org.

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