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Transverse ^{1}H Cross Relaxation in ^{1}H–^{15}N Correlated ^{1}H CPMG Experiments

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Transverse ^{1}H cross relaxation was observed in Carr–Purcell–Meiboom–Gill (CPMG) experiments by recording ^{15}N–^{1}H correlated spectra of amides in HIV protease that was perdeuterated at nonexchangeable sites. Perdeuteration suppresses ^{1}H–^{1}H J coupling and improves spectral resolution and sensitivity. Measurements of cross-peak intensities, arising from cross relaxation, were made as a function of (i) Δf, the frequency difference between the spins, and (ii) τ_{CPMG}, one-half of the duration between CPMG π pulses. Cross peaks were observed when τ_{CPMG} was less than $1/(2\Delta f)$, in agreement with theoretical calculations.

Key Words: **NMR; transverse relaxation; CPMG; perdeuterated protein; spin lock.**

Although transverse cross relaxation was observed in ^{1}H Carr–Purcell–Meiboom–Gill (CPMG) experiments in pioneering work by Vold and Chen in 1972 (*1*), the effects of this phenomenon are difficult to identify in homonuclear experiments. In contrast, we show herein that effects of ^{1}H transverse cross relaxation are clearly evident in ^{1}H–^{15}N correlated ^{1}H CPMG spectra of a ^{15}N-labeled perdeuterated protein, the HIV protease. Direct evidence of transverse cross-relaxation is provided by the appearance of cross peaks, in addition to the main peaks, in the 2D ^{1}H–^{15}N correlated spectra. The two types of peaks allow us to separately monitor auto and cross relaxation. Furthermore, because numerous proton pairs generate cross peaks, we can determine how their chemical shift differences and the timing of the CPMG π pulses affect cross-peak intensity. These measurements then allow us to compare the cross-peak intensities predicted by detailed theoretical calculations with experiment, in the large molecule limit.

We measured ^{1}H–^{15}N correlation spectra of ^{15}N-labeled and perdeuterated HIV protease (the autolysis-resistant, fully active triple mutant, Q7K, L33I, and L63I) bound to the inhibitor DMP323 (*2*), applying either a 2-kHz B_1 field ($R_{1\rho}$) or a CPMG pulse train (R_2), after t_1 evolution. Sixty-four scans were accumulated at each of 100 complex points in t_1, requiring ca. 10 h to measure each 2D spectrum. By dissolving the perdeuterated protein in 95% H_2O, amide and other exchangeable sites are nearly completely protonated, whereas nonexchangeable sites (primarily aliphatic and aromatic carbons) are ca. 85% deuterated. Deuteration simplifies analysis and increases resolution and sensitivity (i) by confining cross relaxation to the amide protons, (ii) by suppressing ^{1}H–^{1}H J coupling, and (iii) by increasing the transverse relaxation times of the amide protons. As discussed previously (*3*), the auto-relaxation rate, ρ_2, of most amide ^{1}H spins is determined primarily by the ^{1}H–^{15}N dipolar interaction (ρ_2 ca. 10 s^{-1}). ^{1}H–^{1}H dipolar interactions also contribute significantly to the relaxation of some spins. The amount varies greatly ($0 < \rho_2 < 15$ s^{-1}) depending upon the surrounding ^{1}H density. Typically, the auto- and cross-relaxation rates for individual proton pairs, ρ_2 and σ_2 respectively, are less than 5 s^{-1} under the conditions used in our experiments.

A portion of a ^{1}H–^{15}N correlated ^{1}H ROESY spectrum, recorded with a 48-ms mixing time, is shown in Fig. 1A. Small ROE cross peaks having opposite signs from the main peaks are observed. These signals are a consequence of magnetization transfer from ^{1}H nuclei, attached to ^{15}N nuclei that evolve in F_1, to ^{1}H nuclei that evolve in F_2. A total of 28 cross peaks was observed, and examination of the crystal structure of the protease showed that the internuclear distance of every proton pair giving rise to a cross peak was less than 5 Å. Because the mixing time is relatively short and the interproton distances are typically greater than 2.4 Å, the intensities of cross peaks are less than 10% of the main peaks.

It was observed that some of the cross peaks remained when the spin lock was replaced by a CPMG sequence, Fig. 1B, with $\tau_{CPMG} = 1$ ms, where τ_{CPMG} is one-half of the duration between π pulses. Comparison of Fig. 1A with Fig. 1B clearly demonstrates that most cross peaks in the CPMG experiment are observed for proton pairs having chemical shifts separated less than ca. 250 Hz. This observation makes qualitative sense,

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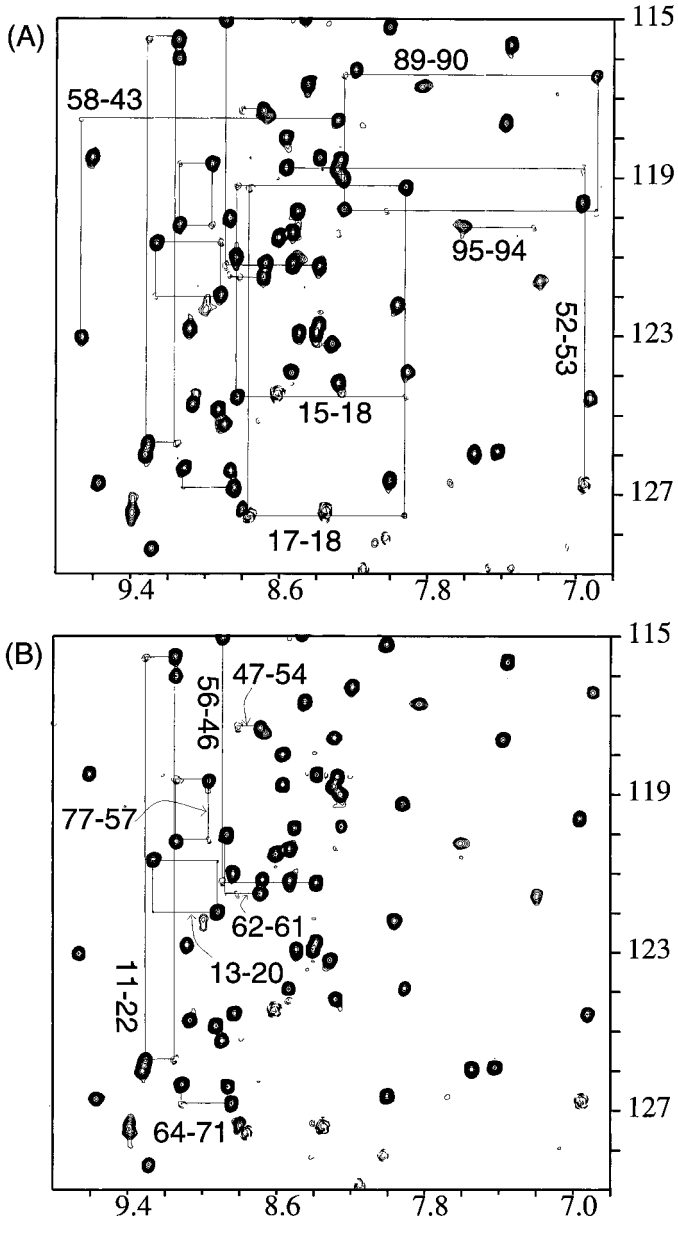

FIG. 1. ^{1}H–^{15}N correlation spectra of ^{15}N-labeled perdeuterated HIV protease bound to DMP323, detected after application of either (A) a 48-ms B_1 field of 2 kHz ($R_{1\rho}$) or (B) a 48-ms CPMG pulse train with τ_{CPMG} = 1 ms. Positive peaks are drawn using solid lines whereas negative peaks are drawn using dashed lines. Experiments were performed using a Bruker DMX 500 MHz spectrometer at 20°C as described previously (*3*).

since in the absence of a B_1 field, proton pairs having a large frequency difference, Δf, will accumulate a large phase difference before the π pulse is applied. As noted previously (*1*, *4*), transverse cross relaxation is effectively quenched for spin pairs whose transverse magnetization components sample all relative phases. Hence, we expect that only spin pairs for which $\Delta f\ \tau_{CPMG}$ is small, and which undergo little relative dephasing, (they can be thought of as roughly locked along a common axis by the π pulses), will exhibit cross peaks.

The relationship between Δf, τ_{CPMG}, and the observed cross-peak intensities was determined experimentally by recording ^{1}H–^{15}N correlated CPMG R_2 spectra using a series of τ_{CPMG} values, and is summarized in Table 1. Examination of the table reveals that cross peaks were only observed when the condition $\tau_{CPMG} < 1/(2\Delta f)$ was satisfied. Furthermore, the strongest cross peaks were observed for spin pairs satisfying the condition $\tau_{CPMG} < 1/(4\Delta f)$, while weak cross peaks were observed when $\tau_{CPMG} > 1/(4\Delta f)$. For example, cross peaks of proton pairs having Δf up to 500 Hz were observed at τ_{CPMG} = 1 ms, but the most intense cross peaks were observed when $\Delta f <$ 250 Hz.

The simplest theoretical description of transverse cross re-

TABLE 1
List of Residue Pairs for Which Cross Peaks Were Observed in Spin-lock and CPMG Experiments

A pair of residues[a]	Chemical shift difference Δf (Hz)	Spin lock B_1 = 2 kHz	CPMG τ_{CPMG} (ms) 1/4 τcpmg (Hz)	0.5 500	1.0 250	3.0 83.3
64–71	60	○		○	○	○
6–7	80	○		○	○	×w
57–77	100	○		○	○	○
61–62	100	○		○	○	○
92–93	110	○		○	○	w
62–73	142	○		○	○	w
93–94	150	○		○	○	×
95–94	160	○		○	○	×
45–56	160	○		○	○	×
13–20	190	○		○	○	×
96–98	193	○		○	○	×
72–73	244	○		○	×w	×
68–69	290	○		○	○	×
32–84	340	○		○	×	×
67–68	370	○		○	×w	×
17–18	380	○		○	×	×
29–30	390	○		○	×	×
66–69	410	○		○	×	×
16–17	422	○		○	×	×
15–18	469	○		○	×	×
3–97	550	○		×w	×	×
89–90	590	○		○	×	×
27–28	600	○		×w	×	×
51–52	650	○		×	×	×
91–92	690	○		×	×	×
43–58	704	○		×	×	×

Note. ○, ×, or w indicate a cross peak observed ($S/N > 4$), not observed, or weakly observed ($2 < S/N < 4$), respectively. xw indicates a tentative peak assignment with $S/N < 2$.

[a] A residue pair "A–B" denotes a cross peak from ^{15}N-labeled *A* to ^{1}H-detected *B*.

laxation is provided by a two-spin system satisfying the following equations of motion (*1*, *4*):

$$dI_i(t)/dt = -\rho_{2i}I_i(t) - \sigma_2 I_j(t) + i2\pi f_i I_i(t) \quad [1]$$

$$dI_j(t)/dt = -\sigma_2 I_i(t) - \rho_{2j}I_j(t) + i2\pi f_j I_j(t). \quad [2]$$

Note that these equations differ from those of Ref. (*5*) only by the inclusion of the terms in f_i and f_j, describing the free precession of spins i and j, respectively. Relaxation mechanisms, in addition to the mutual ^{1}H–^{1}H dipolar interaction, are included in the auto-relaxation rates, so that ρ_{2i} need not equal ρ_{2j}. Note that on account of the amide ^{1}H–^{15}N J coupling, the proton transverse magnetization precesses at two frequencies, $f_i \pm J_{NH}/2$, and in fact evolves as in-phase and antiphase magnetization. However, $\tau_{CPMG}J_{NH}/2 < 0.14$ in our experiments, and the ^{15}N longitudinal relaxation rate is much smaller than the ^{1}H transverse relaxation rate, so that the antiphase signal is smaller than the in-phase component and its relaxation rate is nearly the same as the in-phase component. Hence the J_{NH} coupling affects relaxation primarily by modifying the effective value of Δf, and for this reason only chemical shift precession is taken into account in the calculations.

Equations [1] and [2] were solved using a matrix method (*1*) and Fig. 2 shows the calculated cross-peak intensity, I_{ij}, plotted as a function of τ_{CPMG}. Note that I_{ij} is calculated when each echo is refocused, because the signal is recorded under such a condition in an actual experiment. Figure 2 shows that the cross-peak intensity rapidly decreases when τ_{CPMG} increases beyond $1/(4\Delta f)$, and vanishes when $\tau_{CPMG} = 1/(2\Delta f)$. The cross peak vanishes at this point because the two components of transverse magnetization accumulate a phase difference of 180° during the time interval τ_{CPMG}. Hence, when τ_{CPMG} becomes larger than $1/(2\Delta f)$ the sign of the cross-peak changes (Fig. 3). Because of limited signal-to-noise, we did not observe cross peaks having positive intensity.

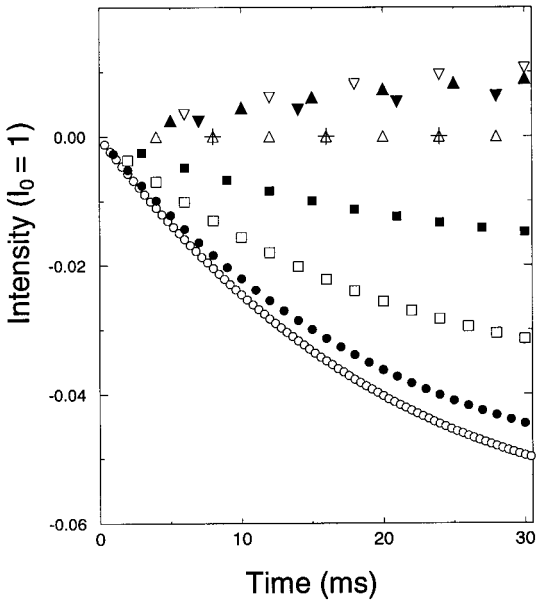

FIG. 2. Calculated time course of CPMG transverse cross-relaxation peak intensities of proton pairs. The difference in chemical shift, Δf, was assumed to be 250 Hz for each pair; ρ_{2i}, ρ_{2j}, and σ_2 were set to 20, 20, and 3 s^{-1}, respectively; τ_{CPMG} values were, 0.2 ms (○), 0.5 ms (●), 1.0 ms ($=1/(4\Delta f)$, □), 1.5 ms (■), 2.0 ms ($=1/(2\Delta f)$, △), 2.5 ms (▲), 3.0 ms ($=3/(4\Delta f)$, ▽), 3.5 ms (▼), and 4.0 ms ($=1/\Delta f$, +). See text for further discussion.

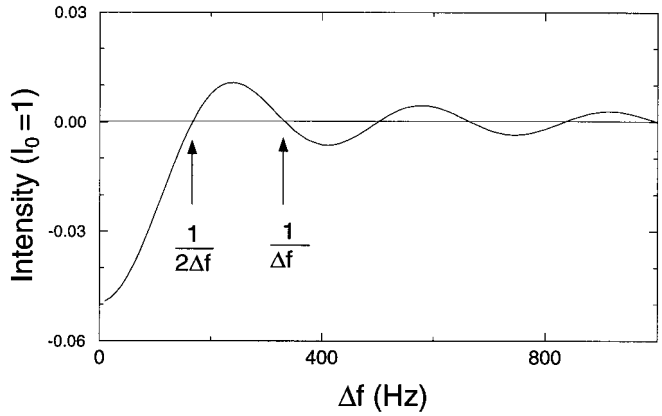

FIG. 3. Calculated Δf dependence of the cross-peak intensity. ρ_{2i}, ρ_{2j}, σ_2, t, and τ_{CPMG} were set to 20 s^{-1}, 20 s^{-1}, 3 s^{-1}, 30 ms, and 3 ms, respectively.

Using Eqs. [1] and [2] together with the assumptions that $\rho_{2i} = \rho_{2j} = \rho_2$, $[\sigma_2/(2\pi\Delta f)]^2 \ll 1$ and Eq. B.4 of Ref. (*6*), it can be shown that

$$I_{ij} = -\sigma_2 t I_0 \exp(-\rho_2 t)\sin(2\pi\Delta f\tau_{CPMG})/(2\pi\Delta f\tau_{CPMG}), \quad [3]$$

where $t = 2n\tau_{CPMG}$ ($n = 1, 2, 3 \ldots$). This expression was verified by numerical calculations. The numerical calculations and the analytical derivation of Eq. [3] showed that a quadrature (imaginary) component of I_{ij} also develops in the CPMG experiment. This component is not observed in our spectra because it is eliminated by a gradient that is applied after the CPMG portion of the pulse sequence (*3*). A quadrature component does not develop in the spin lock experiment.

Equation [3] is consistent with the expression for R_2, derived in the extreme narrowing case by Vold and Chan (*1*) for two dipolar coupled protons. Although the observations presented herein could have been anticipated, based upon the work of Vold and Chan, the numerous well-resolved main peaks and cross peaks in the ^{1}H–^{15}N correlated spectra manifest the transverse cross relaxation much more clearly than was possible in the 1D experiments. In addition, the numerous protein spin pairs that exhibit cross-peaks allow one to measure the dependence of the transverse cross relaxation on both τ_{CPMG} and Δf. Finally our results enable us to confirm the predictions of theory in the large molecule limit, $(\omega_H\tau_c)^2 \gg 1$.

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