Transverse ^{13}C Relaxation of CHD_2 Methyl Isotopmers To Detect Slow Conformational Changes of Protein Side Chains

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Conformational fluctuations of proteins on the ms−μs time scale are often manifest as chemical exchange in NMR spectra.[1] These slow motions are candidates for rate-limiting steps in various biological processes such as substrate binding, product release, and target recognition. Numerous studies of chemical exchange of the protein backbone have been performed using CPMG and spin-lock relaxation rate measurements.[2−6] In contrast, studies of side chain dynamics in proteins have been limited to subnanosecond internal motions,[7−11] except for studies of slow 180° flips of aromatic rings.[12,13] Methyl groups are of prime interest because of their important roles in the formation of hydrophobic cores and in molecular recognition through hydrophobic interactions. However, analysis of methyl ^{13}C transverse relaxation of proteins in solution is complicated by $^{13}C-^{13}C$ *J*-coupling and $^{1}H-^{13}C$ dipole−dipole cross correlation. Although the former problem was solved using methyl ^{13}C specific labeling,[7,14] this approach does not eliminate the effect of cross correlation on transverse ^{13}C relaxation.[15] An elegant alternative approach to study methyl dynamics is ^{2}H relaxation measurements of $^{13}CH_2D$ isotopmers.[16] However, the large quadrupolar R_2 and small chemical shift range of ^{2}H preclude detection of chemical exchange using this method.

Herein, we overcome the problem of dipolar cross correlation by expressing protein in bacteria grown on a 99.9% D_2O medium containing protonated 3-^{13}C-pyruvate,[14,17] and measuring transverse ^{13}C relaxation (R_2) of $^{13}CHD_2$ isotopmers. In this approach, only methyl carbons are ^{13}C labeled and are partially protonated. Other side chain carbon and most hydrogen nuclei are ^{12}C and ^{2}H, respectively, and are not observed in $^{1}H-^{13}C$ HSQC spectra.

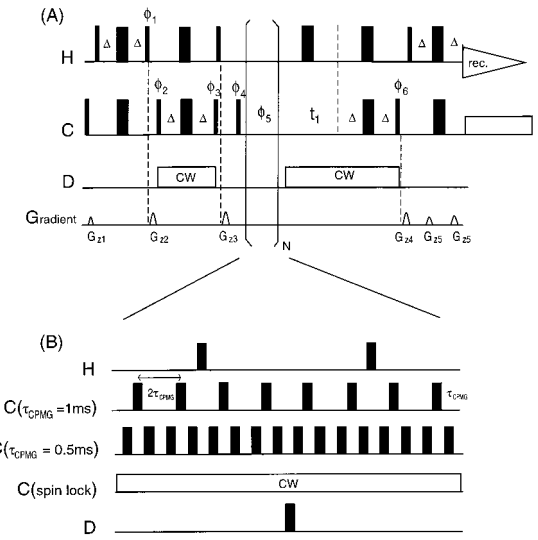

Figure 1. Pulse sequences for measuring ^{13}C R_2 of $^{13}CHD_2$ isotopmers. Narrow and wide black rectangles depict 90 and 180° pulses, respectively. Open rectangles in part A indicate either 2.5 kHz ^{13}C GARP or 0.6 kHz ^{2}H CW decoupling. All pulses have *x* phase unless otherwise indicated. The phase cycle was, $\phi1 = y, -y$; $\phi2 = x, x, -x, -x$; $\phi3 = y$; $\phi4 = -y$; $\phi5 = 4(x), 4(-x)$; receiver $= x, -x, -x, x$. Quadrature in t_1 was achieved by States-TPPI of $\phi6$. (Note that a semiconstant t_1 period, containing a single ^{1}H π pulse, could be used and ^{2}H decoupling during Δ is optional.) ^{1}H and ^{13}C 90° pulse widths were 7.5 and 14 μs, respectively, and Δ was set to 1.92 ms. Sine shaped gradients with amplitudes of 30 G/cm (G_{1z}, G_{4z}), and 25 G/cm (G_{2z}, G_{3z}, G_{5z}) were applied for 0.75 (G_{1z}), 1.5 (C_{2z}), 0.8 (G_{3z}), 0.75 (G_{4z}), and 0.3 (G_{5z}) ms. ^{13}C R_2 values were measured using (i) CPMG sequences with $\tau_{CPMG} = 1.0$ and 0.5 ms, corresponding to effective field strengths of 276 and 551 Hz, respectively,[23] and (ii) a 2.5 kHz spin-lock. The off-resonance effect in the spin-lock experiment was corrected using ^{13}C R_1 data. Spectra were recorded on a Bruker DMX500 at 20 °C using relaxation delays of 16, 32, 64, 96, 128, and 160 ms, with a recycle delay of 2.8 s. Sixteen scans were accumulated for each fid, requiring ca. 20 h to measure R_2 values at one effective field. Spectral widths were 1760.6 (F_1) and 6009.6 (F_2) Hz, and 128 and 512 complex points were collected. Data were processed using nmrPipe.[24]

Among the observable methyl isotopmers, we selected $^{13}CHD_2$ for R_2 measurements for the following reasons: (i) the ^{13}C R_2 value is small because the ^{13}C nucleus is relaxed almost exclusively by a single attached proton (dipolar relaxation by both deuterons contributes only 12% to R_2), (ii) $^{13}C-^{1}H$ dipolar cross-correlation does not contribute to relaxation,[18] (iii) direct $^{13}C-^{13}C$ *J* and dipolar couplings are absent.[14] Thus, the small R_2 values of CHD_2 are sensitive to chemical exchange and are straightforward to analyze.

Since only methyl positions are labeled by ^{13}C, transverse relaxation of CHD_2 methyl groups was measured using refocused-INEPT transfer without a constant time (for $^{13}C-^{13}C$ decoupling) period (Figure 1). After the first INEPT transfer, the product operator C_xH_z evolves differently for CHD_2, CH_2D, and CH_3, becoming C_y, C_xH_z, and $C_yH_zH_z$, respectively, at the end of the second INEPT transfer.[19] ^{13}C and ^{1}H 90° pulses, followed by a gradient pulse, purge multiple quantum coherence stemming from $^{13}CH_2D$ and $^{13}CH_3$ groups. Note that while the CH_3 zero quantum

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(1) Although chemical exchange is a more general phenomenon than conformational exchange, for convenience we use the terms interchangeably.
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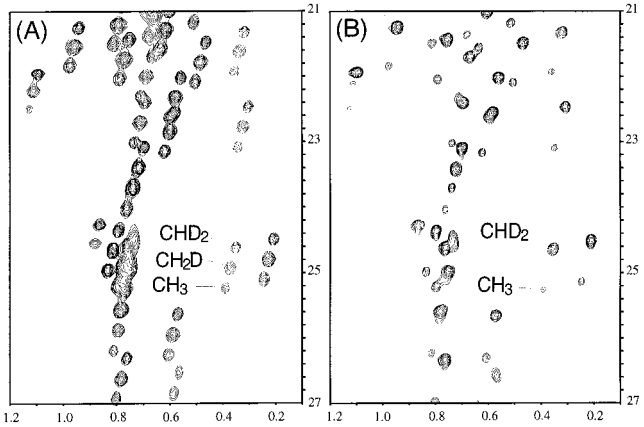

Figure 2. Portions of spectra of HIV-1 protease bound to DMP323 from (A) the $^1H-^{13}C$ HSQC spectrum and (B) a filtered R_2 spectrum. The fully active, isotope labeled, HIV-1 protease variant contains 5 mutations: Q7K, L33I, L63I, C67A, and C95A. The NMR sample (280 μL in a Shigemi microcell) contained ca. 0.6 mM protease dimer in 20 mM phosphate buffer, pH 5.8, in D_2O. In part A, due to the H/D isotope effect, three peaks corresponding to CHD_2, CH_2D, and CH_3 are observed, while in part B, only CHD_2 signals and weak CH_3 signals remain, as discussed in the text.

coherence is not purged, it does not evolve into detectable signal. However, the pulse scheme does not completely suppress $^{13}CH_3$ signals, because in the slow tumbling limit $^{13}C-^1H$ dipolar cross correlation causes the transitions within the $^{13}CH_3$ spin 3/2 manifold to relax at different rates.[15] Despite the imperfect filtering, signal overlap is not severe because the $^{13}CH_3$ signals are weak and sharp. ^1H and ^2H 180° pulses were alternately applied during the CPMG or spin-lock period to suppress any small cross correlation between $^{13}C-H$ dipole, $^{13}C-D$ dipole, and ^{13}C CSA interactions. The effect of ^2H spin flips is negligible for $\tau_{CPMG} < 1$ ms because the methyl $^{13}C-^2H$ *J*-coupling and the ^2H R_1 are small, ca. 20 Hz and ca. 12 s^{-1}, respectively.

We applied the filtered R_2 experiment to the HIV-1 protease homodimer bound the potent inhibitor DMP323 purified as described previously.[20,21] Although small CH_3 signals appear in Figure 2, even at 500 MHz there are few signal overlaps and these are significantly reduced at higher field strengths. Higher fields also significantly increase sensitivity, because methyl ^{13}C and ^1H T_1's do not increase strongly as field strength increases, due to rapid methyl rotation.

Decay curves of the filtered R_2 experiments were well fitted by single-exponential functions (Figure 3). The average R_2 value of all methyl carbons, excluding those undergoing exchange, is 6.5 ± 0.5 s^{-1} (Figure 4). Figure 4 shows that methyl carbons of I3 $C_{\gamma 2}$, L5 $C_{\delta 1}$, and L76 $C_{\delta 2}$/L97$C_{\delta 1}$ have significant chemical exchange contributions to R_2. Although resonances of L76 $C_{\delta 2}$ and L97 $C_{\delta 1}$ are not well resolved, their signal lacks a narrow component and we therefore tentatively conclude that both undergo chemical exchange. In the cases of L5, L76, and L97, chemical exchange contributed to relaxation of only one of the methyl groups (either $\delta 1$ or $\delta 2$). We think that it is likely that both $\delta 1$ and $\delta 2$ methyl groups are mobile, but because their mobility affects their chemical shifts differently, only one methyl carbon (either $\delta 1$ or $\delta 2$) has an observable chemical exchange contribution.

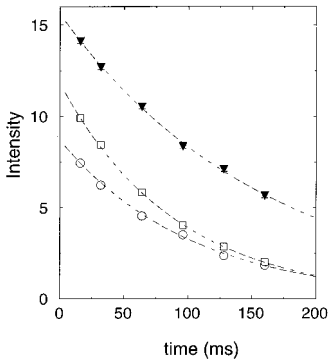

Figure 3. Comparison of three decay curves from the filtered R_2 experiment recorded with $\tau_{CPMG} = 0.5$ ms: ○, I3 $C_{\gamma 2}$; ▼, V11 $C_{\gamma 2}$; □, A28 C_β .

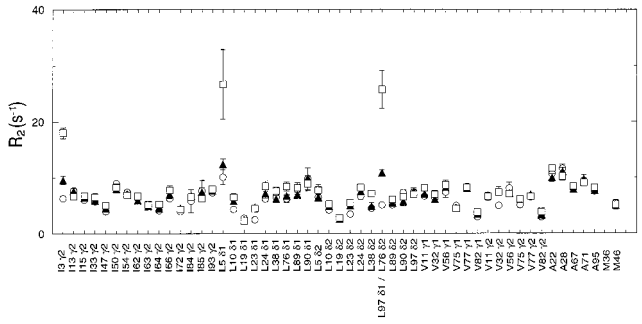

Figure 4. Plot of CHD_2 ^{13}C R_2 values of HIV-1 protease measured using 2 kHz spin-lock (○) or CPMG sequences (see Figure 1) with $\tau_{CPMG} = 0.5$ ms (▲) and $\tau_{CPMG} = 1.0$ ms (□). Note that Ile $C_{\delta 1}$ and Thr C_γ were not significantly labeled,[14] and that the R_2 of M36 C_ϵ at $\tau_{CPMG} = 0.5$ ms is absent because it was too small to be reliably fitted by an exponential decay function.

Methyls I3 $C_{\gamma 2}$, L5 $C_{\delta 1}$, and L97 $C_{\delta 1}$ are located within or adjacent to the β-sheet dimer interface formed by the four terminal β-strands. As described by Ishima et al.,[20] we used the fast-limit exchange equations, together with the average value of the ratio $Q = [R_2(276\text{ Hz}) - R_2(552\text{ Hz})]/[R_2(276\text{ Hz}) - R_2(2\text{ kHz})] = 0.77 \pm 0.08$ for these four residues, to calculate that the exchange correlation time of their mobile side chains is >1ms. Our previous finding of backbone fluctuations in this β-sheet interface was used to rationalize unexplained kinetics of protease maturation.[20] However, backbone flexibility of the β-sheet interface was unexpected because structural studies[21,22] have shown that this interface is well ordered. Hence, chemical exchange of the methyl groups reported herein provides important confirmatory evidence for β-sheet flexibility that may play a role in protease maturation.

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