^{1}H-Detected NMR Relaxation of Methylene Carbons via Stereoselective and Random Fractional Deuteration[1]

Diana M. Kushlan and David M. LeMaster*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University Evanston, Illinois 60208

Received June 9, 1993

With the availability of 2D ^{1}H-detected ^{13}C relaxation experiments for methine[2] and methyl[3] spin systems, the lack of a corresponding technique for measuring the dynamics of methylene carbons represents one of the major impediments to carrying out complete relaxation analyses of macromolecules. The cross correlation effects between ^{1}H–^{13}C dipolar interactions[4] cannot be as readily suppressed for methylene resonances as they can for the symmetric I_3S methyl system.[3] We report here the use of deuterium enrichment as a means of converting a methylene spin system into a pair of independent ^{1}H–^{13}C and ^{2}H–^{13}C dipolar systems for relaxation analysis.

High-power ^{2}H decoupling has recently been applied to ^{1}H-detected heteronuclear experiments as a means of narrowing ^{13}C resonances in deuterated macromolecules via suppression of the ^{2}H scalar interaction.[5] For the AIS system of monodeuterated methylene resonances, ^{2}H decoupling suppresses cross correlation between the ^{1}H–^{13}C and ^{2}H–^{13}C dipoles. As a result, for both longitudinal[4a,6] and transverse[4b,7] ^{13}C relaxation, the ^{1}H–^{13}C dipole can be treated as an IS system having a small ^{13}C{^{2}H} NOE perturbation of the steady state magnetization.

To test this approach in the measurement of ^{1}H-detected ^{13}C T_1 and T_2 values for [2-^2H_R,2-^{13}C]glycine-labeled samples of *Escherichia coli* thioredoxin (MW = 11.7 kDa), previously published experiments[8] based on a refocused INEPT–refocused reverse INEPT sequence were modified to include a 3-kHz WALTZ-modulated ^{2}H decoupling field from the initial INEPT transfer until acquisition. The resultant relaxation rates are given in Table I.

Since a number of protein NMR studies (e.g., refs 5a and 9) have made use of the more readily accessible random fractional deuteration labeling pattern, analogous measurements were carried out on the [50%U-^{2}H,2-^{13}C]glycine-labeled protein. Relaxation experiments on this sample require suppression of the signal from the diprotio methylene resonances. Use of INEPT refocusing periods set to $^1/_2J_{HC}$ in the T_1 pulse sequence used above[8] provides a 25-fold suppression of the I_2S component for a 50 mM [50%U-^{2}H,2-^{13}C]glycine sample. On the other hand, the corresponding pulse sequence for measurement of T_2 gave comparatively little suppression of the diprotio signal. In a modification of this earlier sequence,[8] the CPMG sequence was introduced after the t_1 period of a double-refocused INEPT sequence, which provides I_2S suppression[5b,10] as shown in Figure 1. This pulse sequence provides a 10-fold suppression of the I_2S component with the 50 mM [50%U-^{2}H,2-^{13}C]glycine sample. The corresponding R_2 data for the [50%U-^{2}H,2-^{13}C]glycine-labeled protein sample are given in Table I. Note that this I_2S suppression should also be feasible for ^{15}N relaxation measurements of primary amide positions in mixed $^1H_2O/^2H_2O$ solvents.

Table I. ^{13}C R_1 and R_2 Relaxation Rates from the ^2H_R–$^{13}C_\alpha$ Crosspeaks of [2-^2H_R,2-^{13}C]Glycine- and [50%U-^{2}H,2-^{13}C]Glycine-labeled *E. coli* Thioredoxin at 14.1 T

	R_1 (s^{-1})[a]		R_2 (s^{-1})[b]	
	^2H_R[c]	50%-^2H_R	^2H_R	50%-^2H_R
Gly 21	1.23 (1.4%)[d]	1.06 (2.3%)[e]	32.9 (0.5%)	34.1 (0.7%)[d]
Gly 33	1.22 (2.1%)	1.13 (2.9%)	39.5 (1.0%)	41.3 (4.2%)
Gly 51	1.14 (2.2%)	1.18 (1.5%)	38.0 (1.9%)	34.0 (3.3%)
Gly 65	1.05 (1.9%)	1.16 (1.5%)	40.3 (1.1%)	42.0 (2.2%)
Gly 71	1.10 (0.7%)	1.02 (3.0%)	39.2 (0.6%)	36.9 (1.3%)
Gly 74	1.21 (1.6%)	1.12 (2.0%)	33.3 (1.1%)	32.7 (1.9%)
Gly 84	1.11 (0.8%)	1.11 (3.5%)	36.6 (0.6%)	35.4 (2.7%)
Gly 92	1.17 (1.1%)	1.14 (2.5%)	35.6 (0.8%)	37.4 (3.7%)
Gly 97	1.15 (1.1%)	1.01 (3.3%)	40.8 (0.7%)	40.1 (1.5%)
R_{av}	1.15	1.10	37.4	37.1
R_{av}(^{1}H)	0.95[f]	0.91	35.0	34.7
R_{av}(^{2}H)	0.20	0.19	2.4	2.4

[a] R_1 measured at 0.01, 0.15, 0.25, 0.4, 0.6, 0.9, 1.3, and 1.8 s. [b] R_2 measured at 2.35, 7.06, 11.8, 18.8, 28.2, 42.3, and 58.8 ms for the ^2H_R sample and 2.33, 4.66, 9.32, 14.0, 21.0, 30.3, 41.9, and 55.9 ms for the [50%U-^{2}H] sample. [c] Chiral deuteration of [2-^{13}C]glycine (Cambridge Isotope Labs) using *E. coli* cystathionine γ-synthase.[18] [d] Levenberg–Marquardt nonlinear least-squares exponential fitting.[19] Root-mean-square deviations given in parentheses. [e] Overlap of the ^2H_S and ^2H_R resonances. [f] Relaxation contribution of the ^{1}H–^{13}C dipole (and the ^{2}H–^{13}C dipole) assuming an isotropic 8-ns correlation time.

The close correspondence between the relaxation values for the stereoselective and random fractionally deuterated samples is reinforced by the absence of observable I_2S signals. In addition, the ^{1}H-detected relaxation data closely fit single exponentials, consistent with the absence of dipole–dipole cross correlation effects.

Since chemical shift anisotropy contributions are negligible at this field strength,[11] with the suppression of the ^{2}H scalar interaction, relaxation is anticipated to be purely dipolar. To interpret these relaxation data, it is necessary to partition the individual contributions of the ^{1}H–^{13}C and ^{2}H–^{13}C dipoles. In the extreme narrowing limit, both $R_1(^2H–^{13}C)/R_1(^1H–^{13}C)$ and $R_2(^2H–^{13}C)/R_2(^1H–^{13}C)$ are 0.063, as determined by the $S(S+1)\gamma^2$ factors in the relaxation formula for a single exponential correlation function:

$$R_1(^2H–^{13}C)/R_1(^1H–^{13}C) = \frac{1(2)\gamma_{^2H}{}^2[J(\omega_{^2H}-\omega_{^{13}C}) + 3J(\omega_{^{13}C}) + 6J(\omega_{^2H}+\omega_{^{13}C})]}{^1/_2(^3/_2)\gamma_{^1H}{}^2[J(\omega_{^1H}-\omega_{^{13}C}) + 3J(\omega_{^{13}C}) + 6J(\omega_{^1H}+\omega_{^{13}C})]}$$

The expression for the R_2 ratio is analogous with the additional spectral density functions $4J(0) + 6J(\omega_H)$. At 14.1 T, both the R_1 and R_2 ratios increase to approximately 0.13 for correlation times near the various Larmor frequencies, with the R_1 ratio further increasing to 0.22 and the R_2 ratio returning to 0.063 in the slow tumbling limit. It should be noted that for correlation times above 2 ns, the estimated fraction of R_1 and R_2 due to the ^{1}H–^{13}C dipole varies less than 5%. The ratio R_2/R_1 for the backbone resonances is moderately insensitive to limited-scale

(1) This work was supported by the National Institutes of Health (GM-38779) and the National Science Foundation (DMB-38779).

(2) (a) Nirmala, N. R.; Wagner, G. *J. Am. Chem. Soc.* **1988**, *110*, 7557–7558. (b) Kay, L. E.; Torchia, D. A.; Bax, A. *Biochemistry* **1989**, *28*, 8972–8979.

(3) (a) Palmer, A. G.; Wright, P. E.; Rance, M. *Chem. Phys. Lett.* **1991**, *185*, 41–46. (b) Kay, L. E.; Bull, T. E.; Nicholson, L. K.; Greisinger, C.; Schwalbe, H.; Bax, A.; Torchia, D. A. *J. Magn. Reson.* **1992**, *100*, 538–558.

(4) (a) Werbelow, L. G.; Grant, D. M. *Adv. Magn. Reson.* **1977**, *9*, 189–299. (b) Vold, R. L.; Vold, R. R. *Prog. Nucl. Magn. Reson. Spectrosc.* **1978**, *12*, 79–133.

(5) (a) Grzesiek, S.; Anglister, J.; Ren, H.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 4369–4370. (b) Kushlan, D. M.; LeMaster, D. M. *J. Biomol. NMR*, in press.

(6) Fagerness, P. E.; Grant, D. M.; Kuhlmann, K. F.; Mayne, C. L.; Parry, R. B. *J. Chem. Phys.* **1975**, *63*, 2524–2532.

(7) Vold, R. R.; Vold, R. L. *J. Chem. Phys.* **1976**, *64*, 320–332.

(8) Kay, L. E.; Nicholson, L. K.; Delaglio, F.; Bax, A.; Torchia, D. A. *J. Magn. Reson.* **1992**, *97*, 359–375.

(9) (a) Kalbitzer, H. R.; Leberman, R.; Wittinghofer, A. *FEBS Lett.* **1985**, *180*, 40–42. (b) LeMaster, D. M.; Richards, F. M. *Biochemistry* **1988**, *27*, 142–150. (c) Wang, J.; LeMaster, D. M.; Markley, J. L. *Biochemistry* **1990**, *29*, 88–101.

(10) Bax, A.; Ikura, M.; Kay, L. E.; Torchia, D. A.; Tschudin, R. *J. Magn. Reson.* **1990**, *86*, 304–318.

(11) Palmer, A. G.; Rance, M.; Wright, P. E. *J. Am. Chem. Soc.* **1991**, *113*, 4371–4380.

0002-7863/93/1515-11026$04.00/0

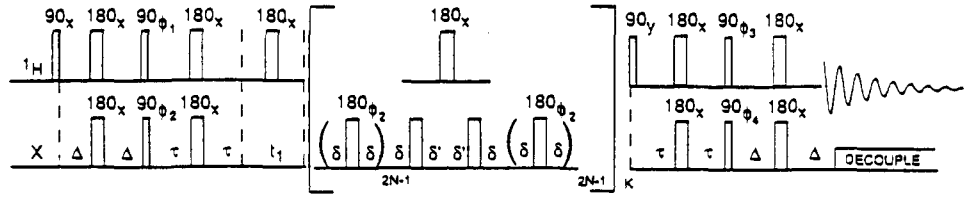

Figure 1. IS spin system selective pulse sequence for measuring T_2 relaxation times. The value of $(2\tau + {}^{13}C_{180°})$ is set to $^1/_2J_{HC}$, and that of Δ is set 20% smaller than τ to minimize relaxation losses. δ is set to 250 μs, while $(2\delta' + {}^1H_{180°}$ pulse$) = 2\delta$. N is chosen so that the ^{1}H pulses in the CPMG sequence are spaced every 5 ms. $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; $\phi_4 = 8(x), 8(-x)$; acq $= x, 2(-x), x, 2(-x, 2(x), -x), x, 2(-x), x$. The phase of the first 180° (X) pulse following the evolution period is inverted every 16 scans. Composite pulses ($90°_x$, $180°_y$, $90°_x$) are used for both this X pulse and for the ^{1}H refocusing pulse in the t_1 period. Quadrature detection is achieved via incrementation of the first heteronuclear 90° pulse according to States et al.[20] When applied to deuterated samples, ^{2}H decoupling is applied starting at the first ^{1}H pulse.

rapid internal motions and can be used to make an initial estimate of a global correlation time[11] of 8 ns.[12]

To provide an independent estimate of the ^{1}H–^{13}C dipole contribution to the overall relaxation, directly observed ^{13}C measurements were carried out on [2-^{13}C]glycine-enriched *E. coli* thioredoxin. R_1 and R_2 values per ^{1}H nucleus of 0.86 and 36.1 s^{-1} were obtained for Gly 33, the only well resolved resonance in a 1D ^{13}C spectrum. These values are within 5–10% of the ^{1}H-detected values, despite the fact that dipole–dipole cross correlation is not suppressed in these direct observe experiments.[13] Since the ^{2}H–^{13}C scalar relaxation affects only the ^{13}C transverse relaxation time,[14] these equivalent R_2 values indicate that the ^{2}H scalar relaxation contribution has been effectively suppressed.

Given that the ^{13}C line widths are approximately 17 Hz and that the one bond ^{2}H–^{13}C isotope shifts are 0.25 ppm[15] (~40 Hz at 14.1 T), the diprotio and monoprotio components are largely resolved. In some cases, INEPT suppression of I_2S systems may prove incomplete due to variation in the $^1H_\alpha$–$^{13}C_\alpha$ coupling constants.[16] On the other hand, several factors can contribute to more effective suppression of the I_2S signal in protein studies. Due the common nonequivalence of the methylene resonances as well as the differential relaxation behavior, the signal from the I_2S component is 4-fold less sensitive than that of the monodeuterated glycine resonances of *E. coli* thioredoxin in HSQC experiments.[5b] Adjustment of the deuteration level can serve to preferentially suppress the diprotio signal. With a [50%U-^{2}H] sample, the proportions of the $^1H_2$ and ^2H_R species are both 25%, while at 75% deuteration, $[^2H_R]/[^1H_2]$ is 3 and the proportion of ^2H_R is 18.75%.

Carbon enrichment will generally be required for protein ^{13}C relaxation analyses using these approaches. The ^{13}C homonuclear magnetization exchange present in uniformly labeled samples is incompatible with the relaxation pulse sequences described to date. However, a highly selective alternating carbon enrichment pattern can be obtained for each of the constituent amino acids in appropriate *E. coli* strains.[17] With the combination of these ^{2}H and ^{13}C labeling approaches, it should prove feasible to determine the relaxation behavior at nearly every site of a biological macromolecule.

(12) The correlation time is concentration dependent, reflecting a mild aggregation at pD 3.9 and 25 °C chosen to mimic the crystallization conditions (Katti, S.; LeMaster, D. M.; Eklund, H. *J. Mol. Biol.* **1990**, *212*, 167–184). The protein concentrations of all samples were carefully matched to 3.5 mM.

(13) Werbelow, L. G.; Grant, D. M. *J. Chem. Phys.* **1975**, *63*, 4742–4749.

(14) Abragam, A. *The Principles of Nuclear Magnetism*; Oxford Univ. Press: Oxford, England, 1961.

(15) Hansen, P. E. *Prog. Nucl. Magn. Reson. Spectrosc.* **1988**, *20*, 207–255.

(16) (a) Egli, H.; Philipsborn, W. v. *Helv. Chim. Acta* **1981**, *64*, 976–988. (b) Vuister, G. W.; Delaglio, F.; Bax, A. *J. Am. Chem. Soc.* **1992**, *114*, 9674–9675. (c) Mierke, D. F.; Grdadolnik, S. G.; Kessler, H. *J. Am. Chem. Soc.* **1992**, *114*, 8283–8284.

(17) LeMaster, D. M.; Cronan, J. E. *J. Biol. Chem.* **1982**, *257*, 1224–1230.

(18) Homer, R. J.; Kim, M. S.; LeMaster, D. M. *Anal. Biochem.*, in press.

(19) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes*, 1st ed.; Cambridge University Press: Cambridge, U.K., 1989.

(20) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286–292.