Assignment of Side-Chain ^{13}C Resonances in Perdeuterated Proteins

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For moderately sized $^{13}C/^{15}N$-labeled proteins, side-chain ^{13}C resonance assignments are obtained from either the HCCH-TOCSY[1] or the HC(CC)(CO)NH[2] experiment. The HCCH-TOCSY has been the experiment of choice because of its extremely high sensitivity and high level of correlative redundancy. The increased number of correlation peaks generated by this experiment, however, may severely aggravate the problem of spectral overlap in larger proteins such as human carbonic anhydrase II (HCA II, 29 kDa, 259-residue monomer) and, in the absence of prior side-chain ^{13}C chemical-shift assignments, may limit the utility of this experiment. Compared to ^1H_N resonances in β-sheet proteins and ^{15}N resonances in general, the dispersion of particular aliphatic ^1H_C and ^{13}C resonances is usually poorer. The HC(CC)(CO)NH experiment would therefore seem more appropriate for the assignment of side-chain ^{13}C resonances in larger proteins. Rapid ^{13}C and ^1H_N T_2 relaxation, however, may render this experiment too insensitive for large proteins. In this regard, the process of sequential backbone assignments in large proteins has benefited greatly from high levels of ^2H incorporation (~85%) at aliphatic sites.[3-5] We have therefore adapted the HC(CC)(CO)NH experiment to perdeuterated proteins by starting the sequence of magnetization transfer steps on ^{13}C[6] instead of ^1H_C. In this communication, we present the results from the C(CC)(CO)-NH experiment on perdeuterated HCA II (^2H-HCA II) and estimate the theoretical increase in sensitivity over the HC(CC)-(CO)NH experiment on protonated HCA II (^1H-HCA II).

We have previously described the preparation of HCA II labeled to 96% in ^2H on aliphatic sites.[7] Figure 1 presents the pulse sequence for the 3D gradient-enhanced and sensitivity-enhanced C(CC)(CO)NH experiment. Since there are no aliphatic protons in the ^2H-HCA II sample, the initial magnetization must originate from the side-chain ^{13}C spins. Figure 2 depicts $^{13}C/^{15}N$ strip plots corresponding to several core residues taken at the ^1H_N chemical shift of the respective $i + 1$ residue. These data allow confirmation of both the sequential $^{13}C_{\alpha/\beta}$ main-chain assignments and the sequential residue type. The S/N of the peaks in Figure 2 ranges from 2:1 for I145-$C_{\gamma 1}$ to 35:1 for A152-C_β. The only correlation missing in Figure 2 is A152-C_α. In general, all Ala $^{13}C_\alpha$ correlations are at best weak in the C(CC)(CO)NH spectrum. A complete analysis of this spectrum has yielded >95% of all previously unassigned side-chain ^{13}C resonances in ^2H-HCA II.

We have estimated the rotational correlation time (τ_c) for HCA II to be ~10.4 ns based on the τ_c value for the *TRP* repressor–DNA complex[5,8] (τ_c ~ 13.4 ns, 37 kDa). The T_2 relaxation times for ^1H_N, ^{15}N, and non-methyl ^{13}C spins have been calculated for ^1H- and ^2H-HCA II.[9-11] The effective ^{13}C relaxation time during the spin lock is that for magnetization, permanently resident on a non-Gly $^{13}C_\alpha$ spin, subjected to a DIPSI-3 pulse train.[12] The non-selective $T_1(^{13}C_D)$ relaxation time for $^{13}C_\alpha$ spins has been qualitatively measured by 1D saturation recovery to be ~2.6 s for ^2H-HCA II. This is significantly shorter than the $T_1(^{13}C_D) = 8.6$ s calculated from intramolecular dipolar and CSA relaxation mechanisms, indicating that these mechanisms do not contribute significantly to ^{13}C relaxation in ^2H-HCA II. The nonselective $T_1(^1H_C)$ is taken as ~1 s; the upper limit for $T_2(^1H_C)$ is set to $T_2(^1H_N)$. These relaxation parameters allow us to estimate the theoretical gain in sensitivity, R_s, for the C(CC)(CO)NH experiment applied to ^2H-HCA II over that for the HC(CC)(CO)NH experiment applied to ^1H-HCA II.

R_s can be factored into a T_2 contribution, $R_s(\{T_2\})$; a T_1 contribution, $R_s(\{T_1\})$; and a scalar coupling contribution, $R_s(\{J\})$:

$$R_s = [\gamma(^{13}C)/\gamma(^1H)]R_s(\{T_2\})\,R_s(\{T_1\})\,R_s(\{J\}) \qquad (1)$$

where { } represents a set of values. $R_s(\{T_1\})$ depends on the recycle delay T_{RC} (1.07 s), $T_1(^{13}C_D)$, and $T_1(^1H_C)$. It is implicit in the calculation of $R_s(\{T_2\})$ and $R_s(\{J\})$ that the various fixed delays have been optimized for the particular experiment on HCA II (^1H vs ^2H).

We have calculated estimates of $R_s(\{T_2\})$, $R_s(\{J\})$, and R_s for rigid-body CD/CH and CD_2/CH_2 aliphatic groups in $^{13}C/^{15}N$-labeled HCA II. $R_s(\{T_1\})$ evaluates to ~0.5 for both groups. $R_s(\{T_2\})$ values are 22.4 and 26.8; $R_s(\{J\})$ values are 1.30 and 2.18; and R_s values are therefore ~3.5 and ~7 for methine and methylene groups, respectively. These results indicate that the C(CC)(CO)NH experiment on ^2H-HCA II is strongly favored on the basis of a large $R_s(\{T_2\})$, which arises due to the increased $T_2(^{13}C_D)$ brought on by perdeuteration. We have also acquired an HC(CC)(CO)NH data set at 600 MHz on a 1 mM protonated $^{13}C/^{15}N$-labeled HCA II sample under equally optimized experimental conditions (data not shown) for comparison with the data in Figure 2. Strip plots from the HC(CC)(CO)NH spectrum analogous to those in Figure 2 show only noise.

Initial estimates of protonated side-chain ^{13}C chemical shifts should facilitate the analysis of the 4D HCCH-TOCSY on ^1H-HCA II. We have been able to reasonably predict the average one-, two-, and three-bond ^2H isotope shifts on $^{13}C_{\alpha/\beta}$ resonances in HCA II. With a ^{13}C resolution of ±0.12 ppm in all cases, the cumulative isotope shifts, which are as large as −1.52 ppm (L140-C_β), have been correctly estimated to within ±0.25 ppm for most $^{13}C_\beta$ resonances. Secondary structure has already been demonstrated to affect the magnitude of the ^2H isotope shift on $^{13}C_\alpha$ resonances.[13] Since secondary structure is known to alter both $^{13}C_\alpha$ and $^{13}C_\beta$ chemical shifts from their random-coil

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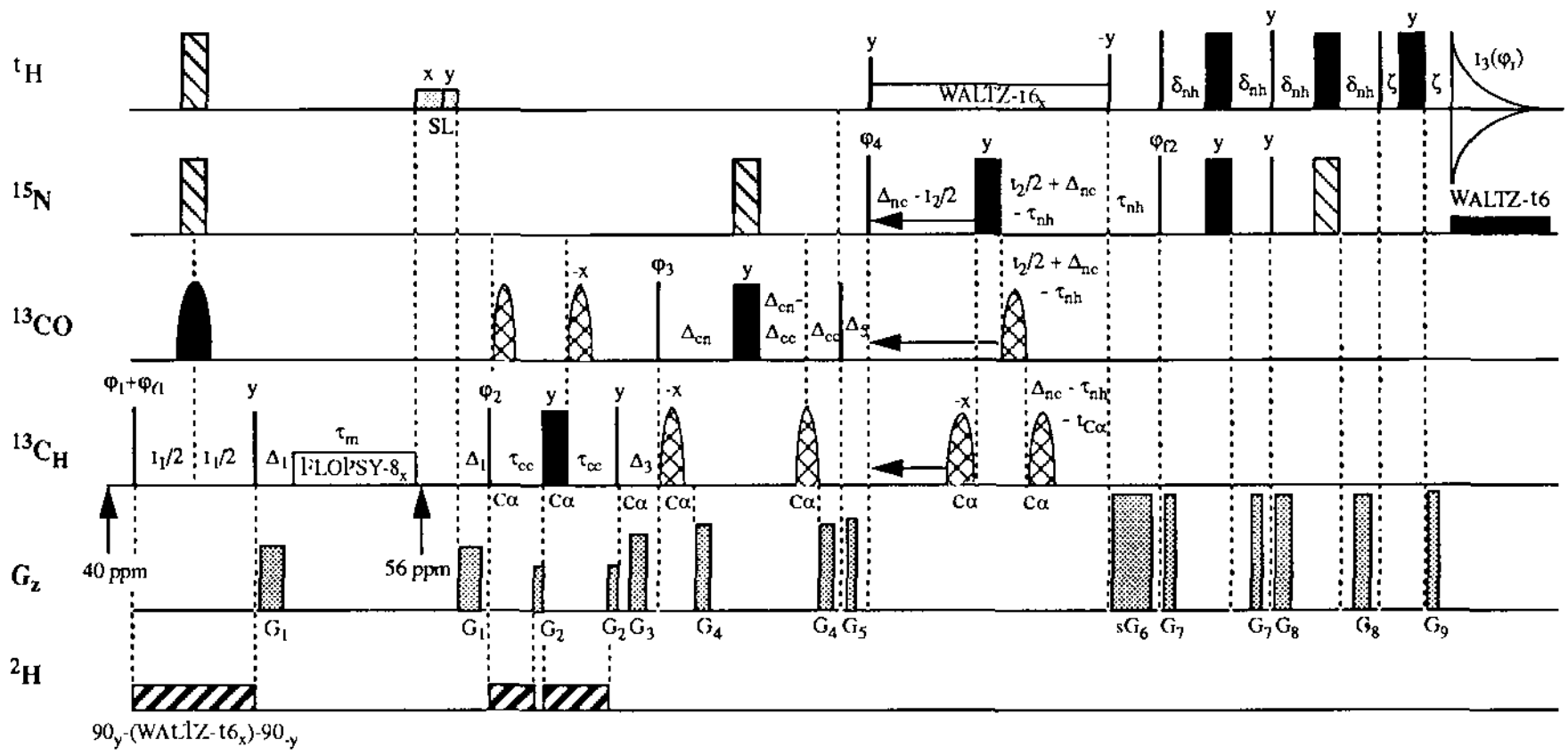

Figure 1. The 3D gradient-enhanced, sensitivity-enhanced C(CC)(CO)NH experiment: 90° pulses are represented by wide lines; simple 180° pulses, by black rectangles; and $90_x240_y90_x$ composite inversion pulses,[15] by striped rectangles. Gaussian- (solid-black) and G$_3$-shaped[16] (cross-hatched) selective inversion pulses are indicated as sinebell-shaped pulses. $^{13}C_H$ pulses selective for $^{13}C_\alpha$ spins are labeled with C$_\alpha$. All other $^{13}C_H$ pulses are nonselective. Complex data were collected in t_1[17] and in t_2,[18] with FIDs for $\varphi_{f1} = (x, y)$ and $\{\varphi_{f2} = (+x,-x)$; $s = (+1,-1)$ on $G_6\}$ being stored separately. States-TPPI[19] was employed on φ_1 and φ_4. The total acquisition time was 70 h. Selected acquisition parameters are as follows: $\gamma B_3(^{13}C_{spinlock}) = 7.94$ kHz with FLOPSY-8,[20] $t_1^{max} = 4.89$ ms, $t_2^{max} = 17.16$ ms, $t_3 = 69.8$ ms, $\tau_m = 20.8$ ms, $\tau_{cc} = 3.0$ ms, $\Delta_{cn} = 13.5$ ms, $\Delta_{nc} = 13.5$ ms, $\Delta_{cc} = 4.55$ ms, $\tau_{nh} = 5.6$ ms, $\delta_{nh} = 2.65$ ms, $\zeta = 1.2$ ms, $G_6 = \pm32.0$ G/cm, $t_{G6} = 5.0$ ms, $G_9 = 32.05$ G/cm, $t_{G9} = 0.5$ ms, and relaxation delay = 1.0 s. The phase cycle is $\varphi_1 = 4(y), 4(-y)$; $\varphi_2 = 8(x), 8(-x)$; $\varphi_3 = 2(x), 2(-x)$; $\varphi_4 = x, -x$; and $\varphi_r = x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, x, 2(-x), x$.

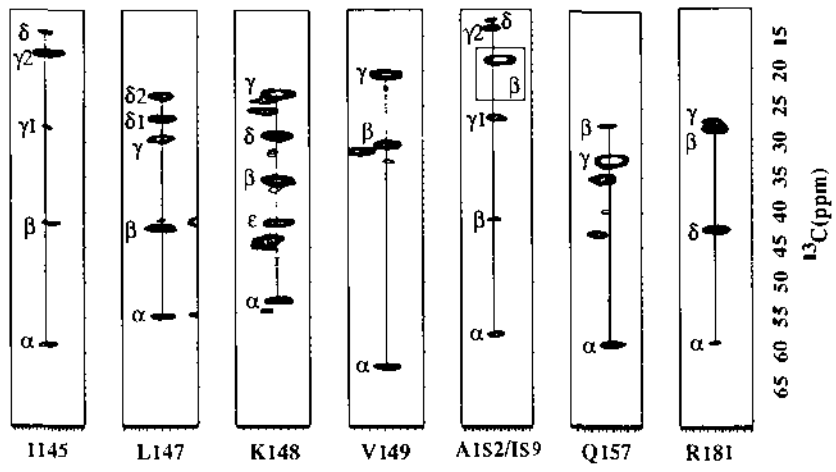

Figure 2. $^{13}C/^{15}N$ strip plots from the C(CC)(CO)NH experiment: Results from several core residues with long side chains demonstrate the efficacy of the method for ^{2}H-HCA II. The side-chain resonances past $^{13}C_{\alpha/\beta}$ are assigned by correlating the i $^{13}C_{\alpha/\beta}$ and $i + 1$ amide $^{15}N/^1H_N$ chemical shifts obtained from the main-chain assignment data with the remainder of the ^{13}C correlations. The A152/I59 strip plot illustrates the ability to determine amino acid types when significant overlap in amide ^{15}N and ^1H_N chemical shifts exists, thereby resolving two otherwise ambiguous main-chain assignments. The boxed correlation peak in this plot is A152-C$_\beta$. These data were acquired at 30.0 °C on a 1.6 mM $^{13}C/^{15}N/^2H$ (96% ^{2}H-labeled) HCA II sample in 100 mM phosphate buffer at pH 6.8. The amide protons were re-exchanged by unfolding the protein and then refolding it by standard methods.[21]

values,[14] the ^{2}H isotope shift on $^{13}C_\beta$ resonances may also be affected by secondary structure. This would tend to decrease the predictive ability of a single, average ^{2}H isotope shift. Side-chain ^{13}C chemical shifts are in general less sensitive to secondary structure and may therefore exhibit a more uniform ^{2}H isotope shift.

We have demonstrated in this communication a method to obtain complete side-chain ^{13}C assignments in ^{2}H-HCA II. On the basis of the number of side-chain correlation peaks and their ^{13}C chemical shifts, one can further restrict the possible residue types for an (^1H_N,^{15}N) pair and thereby increase the level of confidence in the sequential assignments. Prior knowledge of the side-chain ^{13}C chemical shifts in ^{2}H-HCA II will be essential for the assignment of side-chain H_N protons in ^{2}H-HCA II and may also prove to be crucial to the analysis of the 4D HCCH-TOCSY spectrum on ^{1}H-HCA II.

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Supplementary Material Available: Equation used to estimate $R_s(\{T_1\})$ and additional experimental details and parameters (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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