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COMMUNICATIONS

Unambiguous Correlations of Backbone Amide and Aliphatic Gamma Resonances in Deuterated Proteins

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Two 3D NMR pulse sequences that correlate aliphatic gamma carbon resonance frequencies to amide proton and nitrogen chemical shifts in perdeuterated proteins are presented. The HN(COCACB)CG provides only interresidue connectivities ($NH_{(i)}$ and $C_{\gamma(i-1)}$) while the HN(CACB)CG detects both the inter- and intraresidue ($NH_{(i)}$ and $C_{\gamma(i)}$ or $C_{\gamma(i-1)}$) correlations. These two experiments are useful for sequential assignments and the identification of residue type from the C_γ shifts. Spectra acquired on a perdeuterated 53-kDa protein illustrate the sensitivity and utility of these experiments. © 1998 Academic Press

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Assigning chemical shift resonance frequencies is a prerequisite to investigating the structural and dynamic properties of any protein by NMR spectroscopy. Unfortunately, for large proteins having a molecular mass greater than 30–40 kDa, the ability to make unambiguous spectral assignments is challenging for a variety of reasons, including short nuclear spin relaxation times, increased spin diffusion, and greater chemical shift degeneracy. The perdeuteration of proteins has been found to significantly increase the sensitivity for many standard triple-resonance experiments designed to elucidate interresidue correlations that are necessary for backbone assignments (*1, 2*). Sensitivity improvements are largely attributed to significant increases in T_2 relaxation times for carbon nuclei with deuterons substituted for protons (*2*). Protein perdeuteration also decreases the amide proton linewidths due to the reduction in proton–proton spin coupling, leading to a further increase in sensitivity as well as resolution (*3, 4*).

A disadvantage of using perdeuterated protein samples is that numerous pulse sequences that are integral parts of the assignment process are no longer applicable due to deuteration of aliphatic carbons. Experiments which use aliphatic protons as sites of magnetization initiation, frequency labeling, and detection cannot be implemented on perdeuterated protein. Thus, traditional experiments for obtaining intra- and interresidue correlations are limited to those that include the exchangeable amide proton, nitrogen, and carbon chemical shifts.

HC(CC)(CO)NH (11) experiments are generally the experiments of choice for determining (*i*) and (*i*-1) residue types, respectively, because of high sensitivity and large number of chemical shifts obtained which are diagnostic of residue type. Neither of these experiments are applicable to deuterated proteins due to the requirement that aliphatic protons serve as the initial source of magnetization. However, the C(CC)(CO)NH experiment (*12*) circumvents the need to initiate magnetization transfer from aliphatic protons and correlates the amide proton and nitrogen of the (*i*) residue and the aliphatic carbon chemical shifts of the (*i*-1) residue in deuterated proteins. Although the C(CC)(CO)NH experiment has been successfully used on deuterated proteins of 30–40 kDa molecular mass, we found this experiment too insensitive when applied to a perdeuterated 53-kDa protein, glutathione S-transferase (GST).

The need for residue-type information and additional connectivity constraints to aid in the chemical shift assignment process for GST necessitated the development of two new pulse sequences designed to achieve both initiatives. In this Communication, two complimentary 3D heteronuclear NMR experiments which obtain $C_{\gamma(i)}$ and $C_{\gamma(i-1)}$ chemical shifts are described. The two experiments are straightforward extensions of the HN(COCA)CB and HN(CA)CB pulse schemes (*13*) and exploit the long T_2 relaxation times of the aliphatic carbon spins to transfer magnetization from C_β to C_γ. The C_γ chemical shifts complement the C_α and C_β shifts in obtaining residue assignment in three ways. First, the additional pair of sequential inter- and intraresidue connectivities correlate the HN group with the aliphatic gamma carbon (C_γ) resonances of a residue (*i*) and that of the previous residue (*i*-1) in the primary

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sequence of the protein, thus increasing the confidence and extending the length of sequentially linked spin systems. Second, the C_γ chemical shift has a characteristic value for different groups of residues, thus facilitating the identification of residue type on the basis of aliphatic carbon shifts. Third, for those residues without aliphatic C_γ carbons these experiments give rise to negative peaks at the resonance frequency of the last aliphatic position on the side chain, thus providing additional residue type information. For a thorough explanation concerning the origin of the relative sign inversion for the signals from residues without an aliphatic C_β and/or C_γ carbon, see the article by Wittekind and Mueller (*13*).

The first experiment, called HN(COCACB)CG, is diagrammed in Fig. 1a and is used to correlate the amide proton and nitrogen frequencies of one residue (*i*) to that of the C_γ of the previous residue (*i*-1). In this pulse sequence, magnetization is transferred from the NH proton through the CO to the C_α of the previous residue. This is followed by two INEPT-type transfers of magnetization from the C_α to the C_γ carbon. The second experiment diagrammed in Fig. 1b, the HN(CACB)CG, complements the HN(COCACB)CG by correlating the backbone amide proton and nitrogen frequencies to the gamma resonance of both (*i*) and (*i*-1) residues. The HN(CACB)CG pulse sequence is similar to the HN(COCACB)CG experiment. However, the sensitivity of this experiment is expected to be less than half that of the HN(COCACB)CG experiment due to a longer period of transverse magnetization for the nitrogen spin and an additional division of magnetization due to coupling of the N spin to both the (*i*) and (*i*-1) C_α nuclei. Neither experiment detected C_γ signals for Ile or Val residue types due to sensitivity losses resulting from incomplete refocusing of magnetization at the aliphatic branch point. However, this loss can be minimized for these residue types by returning the INEPT periods that create and refocus C_β and C_γ antiphase magnetization and thereby increase the sensitivity of the experiments toward residues that branch prior to the C_γ position (*14*).

The 3D (^{1}H, ^{15}N, ^{13}C) HN(COCACB)CG and HN(CACB)CG experiments were performed on a 0.65 mM (dimer concentration) sample of human mu class glutathione S-transferase (GSTHUM2-2). The protein is a homodimer with a total molecular mass of 53 kDa. The protein was overexpressed and uniformly labeled with ^{15}N, ^{13}C, and ^{2}H in *Escherichia coli* using commercially available media (Cambridge Isotope Laboratories, Cambridge, MA) containing perdeuterated ^{13}C-enriched carbon sources. The protein was isolated as previously described (*15*). Since some amide sites do not exchange under nondenaturing conditions, only 83% of the possible number of amide resonances are observed in the HSQC spectrum. The NMR sample was prepared in water containing 5% D_2O, 50 mM sodium chloride, 1 mM dithiothreitol, 0.02% sodium azide, and 10 mM potassium phosphate buffer at pH 7.0. NMR data were acquired at 600 MHz on a Bruker DMX spectrometer at 25°C.

Figure 2 shows strip plots from the NMR spectra obtained from the HN(CACB)CG and HN(COCACB)CG experiments. For purposes of comparing signal to noise, a region from the HN(CA)CB spectrum is also shown for residue L85 and is plotted with similar contour level increments. The HN(COCACB)CG experiment proved to be quite sensitive even for a 53-kDa protein. Excluding Ile or Val residues and residues that do not exchange amide protons after expression in deuterated media, greater than 76% of expected C_γ and terminal C_β or C_α chemical shifts were observed in the spectrum. Of the remaining residues for which resonance peaks were expected but not observed in the HN(COCACB)CG spectrum, greater than one-half of these also do not show peaks in the HN(COCA)CB experiment. The HN(CACB)CG experiment, as expected, is not as sensitive as the HN(COCACB)CG experiment and 60% of all of the expected intraresidue aliphatic shifts were observed. For these peaks, the signal-to-noise ratio in the HN(COCACB)CG was typically 40 to 50% of that observed in the HN(COCA)CB spectrum. The peak intensity is, in general, correlated with local mobility. For example, residues 88 and 89 have high thermal factors derived from X-ray crystallography for C_α atoms (30 and 26 Å^2, respectively (*16*)) and show intense peaks in the C_γ experiments. However, there are exceptions: Cys 86 is a relatively immobile residue (temperature factor is 17 Å^2) but shows an intense peak.

In addition to frequency correlations, residue-type information is obtained utilizing the C_γ and negative C_α or C_β shifts. For example, the C_β chemical shift for L85 in Fig. 2 could arise from an Asp, Phe, Ile, Asn, Leu, Tyr, or Cys side chain. The C_α shift of 54 ppm (not shown) restricts the assignment to Asp, Phe, Asn, Leu, Tyr, or Cys. The addition of the C_γ shift results in assigning this spin system as Leu. Observations of negative peaks in either the HN(COCA)CG or HN(CACB)CG are also diagnostic of residue type and can be used in an analogous manner. For example the existence of a negative C_β peak for S89 in Fig. 2 readily distinguishes the residue type as Ser from Thr, but this can often be accomplished on the basis of C_β shifts alone.

Figure 2 demonstrates that C_γ interresidue connectivity can be observed in the HN(COCACB)CG experiment, and the intraresidue C_γ shift can be obtained from the HN(CACB)CG experiment. Connectivities between HN groups and their respective C_γ (*i*-1) spins can also be observed in the C(CC)(CO)NH experiment (*12, 17*). In this experiment, the magnetization starts on aliphatic carbon spins, is frequency labeled, and transferred to the α-carbon by an isotropic mixing period (TOCSY). Although the C(CC)(CO)NH experiment can, in principle, also provide aliphatic C_γ shifts, there are several reasons why the C_γ experiments presented here complement the C(CC)(CO)NH experiment. First, the C(CC)(CO)NH experiment can suffer from spectral overlap due to the small chemical shift dispersion for C_β and C_γ carbon resonances for some residues while the C_γ experiments only show one peak. In addition, the C(CC)(CO)NH experiment cannot unambiguously distinguish C_γ resonance signals from other possible aliphatic carbon signals. Second, the C(CC)(CO)NH exper-

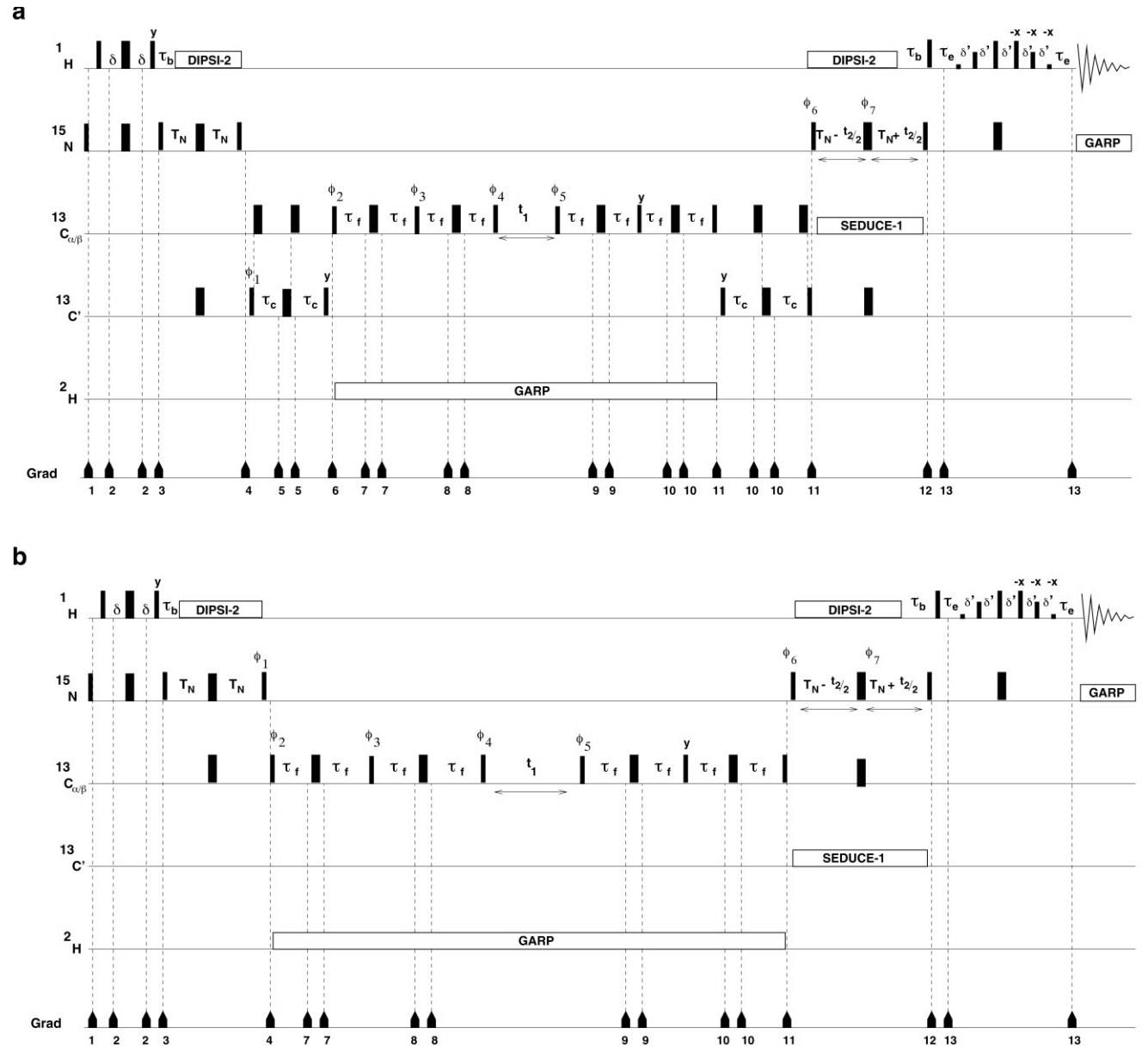

FIG. 1. Pulse schemes of the HN(COCACB)CG (a) and HN(CACB)CG (b) experiments. Narrow and wide rectangular blocks represent RF pulses of 90° and 180° flip angles, respectively. Proton RF pulses were applied at the solvent frequency. The DIPSI-2 scheme (*19*) was used at a 0.5-kHz RF field to decouple protons. Nitrogen decoupling was performed with a GARP-modulated scheme (*20*) at a field strength of 1.2 kHz. The carriers for the $C_\alpha/C_\beta/C_\gamma$ and carbonyl (C′) channels were set at 42.5 and 175 ppm, respectively. Rectangular semi-selective 90° RF pulses were delivered on the carbon channels with pulse widths of 50 and 53 μs, respectively. Carbonyl 180° RF pulses were applied at twice the length of the 90° pulse. The ^{13}C aliphatic inversion pulses were applied at twice the RF power with the same length as the 90° pulses. Carbonyl decoupling was accomplished using a SEDUCE-1 (*21*) decoupling at a field strength of 1.8 kHz. ^{2}H decoupling was performed using a GARP scheme at a field strength of 0.6 kHz centered at 3.5 ppm. The delays used were $\delta = 2.25$ ms, $\tau_b = 5.5$ ms, $T_N = 11.5$ ms (13 ms for the HN(CACB)CG experiment), $\tau_c = 4.2$ ms, $\tau_f = 6.6$ ms, $\delta' = 0.21$ ms, and $\tau_e = (2\delta - 3.5\delta')/2$ ms (*23*). Rectangular gradient pulses were applied at times and power levels as follows: G_z 1 (0.9 ms, −6 G/cm), G_z2 (0.5 ms, 4 G/cm), G_z3 (1 ms, 10 G/cm), G_x3 (1 ms, 16 G/cm), G_z4 (0.9 ms, 4 G/cm), G_z5 (1 ms, 5.5 G/cm), G_z6 (1 ms, 15 G/cm), G_z7 (0.8 ms, 2.5 G/cm), G_z8 (0.8 ms, 7 G/cm), G_z9 (0.9 ms, 8 G/cm), G_z10 (1 ms, 2 G/cm), G_z11 (0.6 ms, 10 G/cm), G_z12 (1 ms, 5 G/cm), G_x12 (1ms, 9G/cm), G_z13 (0.8 ms, 10 G/cm), and G_y13 (0.8 ms, 18 G/cm). The phase cycling was $\phi_1 = x, -x; \phi_2 = x, x, -x, -x; \phi_3 = 4(y), 4(-y); \phi_4 = 8(y), 8(-y); \phi_5 = -y; \phi_6 = x; \phi_7 = 4(x), 4(-x); \phi_{rec} = x, -x, -x, x$. States–TPPI method (*24*) of quadrature detection was used by simultaneously incrementing ϕ_2, ϕ_3, and ϕ_4 for the carbon and ϕ_6 for the nitrogen dimensions, respectively. In these sequences the water magnetization is dephased by a WATERGATE-type sequence (*22*) of six proton pulses with equivalent amplitudes but pulse widths of 0.231, 0.692 1.462, 1.462, 0.692, and 0.231 that of the proton 90° pulse width, respectively, during the last INEPT period. If desired, the water magnetization can be restored via radiation damping, back to the *z* axis by the application of a refocusing gradient after acquisition (amplitude = −2*G13). Lock stability is not affected by the deuterium decoupling on the instrument used for these studies. However, stability could be improved by bracketing each of the decoupling periods with 90°_y and -90°_y pulses.

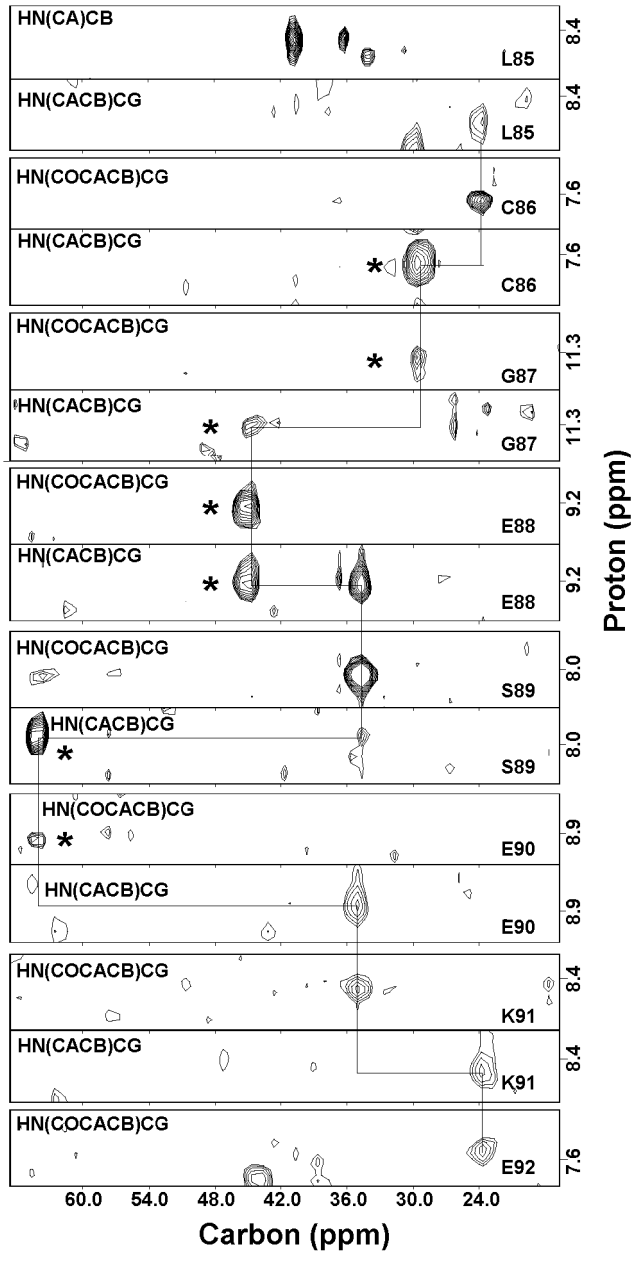

FIG. 2. $^{13}C/^{1}H$ strip plots from NMR spectra obtained from HN(COCACB)CG, HN(CACB)CG, and HN(COCA)CB experiments. Peaks marked with an asterisk are inverted with respect to unmarked peaks of positive amplitude. The width of the strips in the proton dimension is 0.2 ppm. The HN(COCACB)CG data were recorded as a 40 (t_1) × 30 (t_2) × 1024 (t_3) matrix of complex points at spectral widths of 8750, 1850, and 9000 Hz in the carbon (F_1), nitrogen (F_2), and proton (F_3) dimensions, respectively. A total of 80 scans were signal averaged using States–TPPI mode for quadrature detection for both the real and imaginary components of each t_1 and t_2 increment, giving a total acquisition time of just under 6 days. The HN(CACB)CG data set was acquired using 64 scans as a 30 (t_1) × 22 (t_2) × 1024 (t_3) matrix of complex points with the same spectral widths as above. The HN(COCA)CB(2) was acquired with 64 scans as a 40 (t_1) × 30 (t_2) × 1024 (t_3) matrix of complex points at spectral widths of 8750, 1850, and 9000 Hz in the carbon (F_1), nitrogen (F_2), and proton (F_3) dimensions, respectively. The data for all experiments were processed using FELIX 95 software (BIOSYM technologies, San Diego, CA). Residual water signal was removed from each experiment in the directly detected dimension (F_3) by deconvolution in the time domain (*24*). Linear prediction was performed on the three data sets, generating a total number of points equal to approximately one and a half times the acquired number of complex points and zero filled to the processed matrix limits of 128 points in the carbon (F_1) and nitrogen (F_2) dimensions, respectively. Contour levels are drawn at approximately the same intervals for strips from the same experiment. The signal-to-noise ratio for the interresidue C_γ peak in the strip labeled E92 from the HN(COCACB)CG experiment is approximately 5.5 to 1, and the intraresidue C_γ peak in the strip labeled K91 from the HN(CACB)CG is approximately 4 to 1.

iment, when applied to GST, was found to be less sensitive than the C_γ experiments. A direct comparison of signal-to-noise between the C(CC)(CO)NH and the HN(COCACB)CG experiments was difficult due to the weak signal in the C(CC)(CO)NH experiment. Therefore, the relative sensitivities of these two experiments were estimated by comparing the CA(CO)NH experiment to the HN(CO)CA experiment. Using a recycle delay of 2.6 s, the HNCOCA experiment was 10-fold more sensitive than the CA(CO)NH experiment. Decreasing the recycle time to 1.6 s did not significantly alter the relative sensitivities of the experiments. For the complete experiments this sensitivity advantage would be reduced by an estimated two- to threefold for many residue types, because the TOCSY transfer in the C(CC)(CO)NH would be more efficient than the INEPT transfers in the HN(COCACB)CG experiment (*18*). Despite the fact that the HN(COCACB)CG experiment requires additional time for transverse magnetization associated with amide protons (4.5 ms, 30 ms average T_2), amide nitrogen (25 ms, 30 ms average T_2), and aliphatic carbons (20 ms, 28 ms average T_2) than the C(CC)(CO)NH experiment, the sensitivity of the experiment is greater with GST. The increase in sensitivity of the C_γ experiments can be attributed to the efficiency of short recycle times made possible by the short T_1 relaxation time of the amide protons (~1.0 s) relative to the T_1 times for deuterium bound carbon spins (>4.5 s). In addition, for the C_γ experiments, the polarization transfer from the amide proton yields a large increase in the initial nuclear spin polarization (relative to carbon) that is only modestly attenuated during transfer of magnetization out to the C_γ atom due to the long T_2 times at the intermediary deuterated carbon sites.

The experiments presented in this Communication should facilitate the assignment of backbone ^{15}N, H_N, $^{13}C_\alpha$, $^{13}C_\beta$, and $^{13}C_\gamma$ chemical shifts in deuterated proteins. These experiments not only provide an approach to obtain an additional set of sequential connectivities but also provide specific residue-type information for the (*i*) and (*i*-1) residues. The results demonstrate that the reduction in aliphatic carbon relaxation that accompanies perdeuteration can be exploited to transfer magnetization from the amide proton to the gamma carbon in proteins as large as 53 kDa.

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REFERENCES

1. T. Yamazaki, L. Weontae, M. Revington, D. L. Mattiello, F. W. Dahlquist, C. H. Arrowsmith, and L. E. Kay, An HNCA pulse scheme for the backbone assignment of ^{15}N, ^{13}C, ^{2}H-labeled proteins: Application to a 37-kDa TRP repressor-DNA complex, *J. Am. Chem. Soc.* **116,** 6464–6465 (1994).
2. T. Yamazaki, W. Lee, C. H. Arrowsmith, D. R. Muhandiram, and L. E. Kay, A suite of triple resonance NMR experiments for the backbone assignment of ^{15}N, ^{13}C, ^{2}H-labeled proteins with high sensitivity, *J. Am. Chem. Soc.* **116,** 11,655–11,666 (1994).
3. D. A. Torchia, S. W. Sparks, and A. Bax, Delineation of α-helical domains in deutered Staphylococcal nuclease by 2D NOE NMR spectroscopy, *J. Am. Chem. Soc.* **110,** 2320–2321 (1988).
4. D. M. LeMaster, Deuterium labelling in NMR structural analysis of larger proteins, *Quart. Rev. Biophys.* **23,** 133–174 (1990).
5. L. E. Kay, M. Ikura, R. Tschudin, and A. Bax, Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins, *J. Magn. Reson.* **89,** 496–514 (1990).
6. A. Bax and M. Ikura, An efficient 3D NMR technique for correlating the proton and ^{15}N backbone amide resonances with the α-carbon of the preceding residue in uniformly ^{15}N/^{13}C enriched proteins, *J. Biomolec. NMR.* **1,** 99–104 (1991).
7. D. R. Muhandiram and L. E. Kay, Gradient-enhanced triple-resonance three-dimensional NMR experiments with improved sensitivity, *J. Magn. Reson. B* **103,** 203–216 (1994).
8. H. Matsuo, E. Kupce, H. Li, and G. Wagner, Use of selective C$^{\alpha}$ pulses for improvement of HN(CA)CO-D and HN(COCA)NH-D experiments, *J. Magn. Reson. B* **111,** 194–198 (1996).
9. S. Grzesiek, J. Anglister, H. Ren, and A. Bax, ^{13}C line narrowing by ^{2}H decoupling in ^{2}H/^{13}C/^{15}N-enriched proteins. Application to triple resonance 4D J connectivity of sequential amides, *J. Am. Chem. Soc.* **115,** 4369–4370 (1993).
10. A. Bax, G. M. Clore, and A. M. Gronenborn, ^{1}H-^{1}H correlation via isotropic mixing of ^{13}C magnetization, a new three-dimensional approach for assigning ^{1}H and ^{13}C spectra of ^{13}C enriched proteins, *J. Magn. Reson.* **88,** 425–431 (1990).
11. G. T. Montelione, B. A. Lyons, S. D. Emerson, and M. J. Tashiro, An efficient triple resonance experiment using carbon-13 isotropic mixing for determining sequence-specific resonance assignments of isotopically-enriched proteins, *J. Am. Chem. Soc.* **114,** 10,974–10,975 (1992).
12. B. T. Farmer II and R. A. Venters, Assignment of side-chain resonances in perdeuterated proteins, *J. Am. Chem. Soc.* **117,** 4187–4188 (1995).
13. M. Wittekind and L. Mueller, HNCACB, a high sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with alpha- and beta-carbon resonances in proteins, *J. Magn. Reson. B* **101,** 201–205 (1993).
14. A. Bax, M. Ikura, L. E. Kay, D. A. Torchia, and R. Tschudin, Comparison of different modes of two-dimensional reverse-correlation NMR for the study of proteins, *J. Magn. Reson.* **86,** 304–318 (1990).
15. W. R. Vorachek, W. R. Pearson, and G. S. Rule, Cloning, expression, and characterization of a class-mu glutathione transferase from human muscle, the product of the GST4 locus, *Proc. Natl. Acad. Sci.* **88,** 4443–4447 (1991).
16. S. Raghunathan, R. J. Chandross, R. H. Kretzinger, T. J. Allison, C. J. Penington, and G. S. Rule, Crystal structure of human class mu glutathione transferase GSTM2-2. Effects of lattice packing on conformational heterogeneity, *J. Mol. Biol.* **238,** 815–832 (1994).
17. S. Grzesiek, J. Anglister, and A. Bax, Correlation of backbone amide and aliphatic side-chain resonances in ^{13}C, ^{15}N enriched proteins by isotropic mixing of ^{13}C magnetization, *J. Magn. Reson. B* **101,** 114–119 (1993).
18. G. M. Clore, A. Bax, P. C. Driscoll, P. T. Wingfield, and A. M. Gronenborn, Assignment of the side-chain ^{1}H and ^{13}C resonances of Interleukin-1β using double- and triple-resonance heteronuclear three-dimensional NMR spectroscopy, *Biochemistry* **29,** 8172–8184 (1990).
19. A. J. Shaka, C. J. Lee, and A. Pines, Iterative schemes for bilinear operators: Application to spin decoupling, *J. Magn. Reson.* **77,** 274–293 (1988).
20. A. J. Shaka, P. B. Barker, and R. Freeman, Computer-optimized decoupling scheme for wideband applications and low-level operation, *J. Magn. Reson.* **64,** 547–552 (1985).
21. M. McCoy and L. Mueller, Selective shaped pulse decoupling in NMR: Homonuclear [^{13}C]carbonyl decoupling, *J. Am. Chem. Soc.* **114,** 2108–2112 (1992).
22. S. Talluri and G. Wagner, An optimized 3D NOESY-HSQC, *J. Magn. Reson. B* **112,** 200–205 (1996).
23. D. Marion, M. Ikura, R. Tschudin, and A. Bax, Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins, *J. Magn. Reson.* **85,** 393–399 (1989).
24. D. Marion, M. Ikura, and A. Bax, Improved solvent suppression in one- and two-dimensional NMR spectra by deconvolution of time domain data, *J. Magn. Reson.* **84,** 425–430 (1989).