directly after the MLEV-17 mixing time in the 2D ^{13}C TOCSY-REVINEPT experiment. This leads to an experiment in which ^{13}C NMR signals and the scalar correlated ^{13}C spins are detected in ω_1 and ω_2, respectively, and the protons attached to the carbons (ω_2) are detected in ω_3.

Cross-sections (ω_1,ω_3) of a 3D [^{13}C-^{13}C-^1H] TOCSY-REVINEPT spectrum of [^{13}C,^{15}N] T4 lysozyme are shown in Figure 1, B and C. The spectra are markedly simplified compared to the 2D ^{13}C TOCSY-REVINEPT spectra, facilitating the identification of the amino acid spin systems. For example, from the (ω_1,ω_3) plane located at 69.2 ppm (ω_2), the ^{13}C spectra of three amino acid spin systems are resolved and identified as threonines from the characteristic ^{13}C chemical shifts. Since this plane is located at a frequency typical for β-carbons of these residues, the β-proton chemical shifts (ω_3) are also obtained. The shifts of the other protons of the threonine spin systems are determined from planes located at the ω_2 frequencies (equal to the ω_1 frequencies) of the Cα and Cγ carbons. As illustrated for the γ protons in panel C, these proton frequencies can easily be extracted from more crowded planes because they contain the same ^{13}C (ω_1) subspectrum as the β protons (panel B).

In conclusion, 2D and 3D NMR experiments are described that employ isotropic ^{13}C-^{13}C magnetization transfer to provide spin system assignments for *both* ^{13}C and ^1H signals in the spectra of large proteins. These assignments, which cannot be obtained from classical proton 2D experiments, are necessary for the identification of NOEs used in the structure determination of large proteins.

Acknowledgment. This work was supported in part by NIH Grant U01 AI27220-01.

(8) Shaka, A. J.; Keeler, J.; Frenkiel, T.; Freeman, R. *J. Magn. Reson.* **1983**, *52*, 335-338.
(9) Delsuc, M. A.; Lallemand, J. Y. *J. Magn. Reson.* **1986**, *69*, 504-507.

Proton-Proton Correlation via Carbon-Carbon Couplings: A Three-Dimensional NMR Approach for the Assignment of Aliphatic Resonances in Proteins Labeled with Carbon-13

Lewis E. Kay, Mitsuhiko Ikura, and Ad Bax*

Laboratory of Chemical Physics, NIDDK
National Institutes of Health
Bethesda, Maryland 20892

Received September 12, 1989

Complete resonance assignment of the ^1H NMR spectrum of a protein is a prerequisite for obtaining its high-resolution solution structure. For proteins with molecular weights of less than 10 kDa, these assignments often can be obtained by systematic analysis of ^1H-^1H J correlation and NOE spectra. For larger proteins, the ^1H line width is often too large for efficient correlation via the relatively small ^1H-^1H J couplings, seriously impeding the assignment process. Although these problems can be largely solved for the polypeptide backbone by heteronuclear ^{15}N-^1H 3D NMR techniques[1] and selective labeling experiments,[2] these methods are less useful for the amino acid side chains. As recently shown by Markley and co-workers,[3] J_{CC} couplings can be used for the assignments of protein carbon spectra by recording ^{13}C-^{13}C double quantum INADEQUATE spectra. Subsequent heteronuclear correlation then provides proton assignments for resolved resonances. As Markley et al. point out, a relatively low level (<50%) of ^{13}C enrichment is advantageous in the application of this method. Here we present a different approach that requires a high level of enrichment but provides superior sensitivity and resolution.

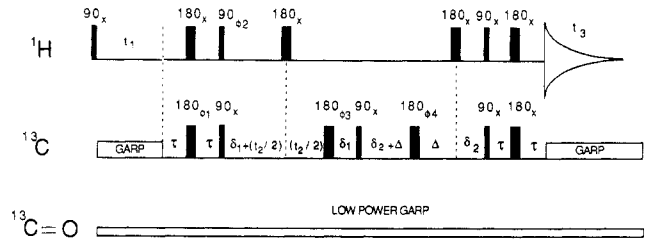

Figure 1. Pulse scheme of the 3D HCCH correlation technique. The delay τ is slightly shorter than $1/(4J_{CH})$, 1.5 ms. The delays δ_1 and δ_2 are $1/(6J_{CH})$ to permit optimal transfer from CH, CH_2, and CH_3 groups simultaneously. By setting δ_1 or δ_2 to a longer value, (CH)/(CH_2,CH_3) editing of the 3D spectrum can be obtained. The duration ($2\Delta + 2\delta_2$) is $1/(4J_{CC})$. The number of ^1H and ^{13}C 180° pulses has been minimized by concatenation, as described elsewhere.[9] The phase cycling is ϕ_1 = 16(x), 16(-x), $\phi_2 = y, -y, \phi_3$ = 2(x), 2(y), 2(-x), 2(-y), ϕ_4 = 8(x), 8(y), Acq. = 2(x,-x,-x,x), 2(-x,x,x,-x). Data are acquired with the TPPI-states method[10] for obtaining quadrature in both the t_1 and t_2 dimensions (by altering the phase of the first 90° ^1H pulse and the first 90° ^{13}C pulse).

We propose to use the relatively large and uniform one-bond ^{13}C-^{13}C J couplings combined with the large heteronuclear $^1J_{CH}$ couplings to transfer magnetization from one proton to its vicinal neighbor. Because $^1J_{CC}$ couplings are relatively uniform (33-45 Hz for sp^3 carbons) the transfer efficiency from one proton to another is independent of conformation, presenting an ideal pathway for assignment of the side chains. Technically, this H→C→C→H approach is most easily executed as a 2D NMR experiment, yielding spectra that appear similar to COSY. However, for larger proteins such 2D spectra show very severe overlap. Also, because all protons observed are attached to ^{13}C, the large heteronuclear dipolar coupling causes substantial proton line broadening, nearly doubling the line width and aggravating the overlap problem. This overlap can be removed by performing the experiment in the 3D mode, separating the "COSY" spectra by the ^{13}C frequency of either the origin CH_n or the destination CH_n site.

One of the two 3D pulse schemes used in our study is sketched in Figure 1. A regular ^1H-^{13}C correlation sequence[4] is used to transfer proton polarization to ^{13}C. The magnitude of polarization transferred is t_1 modulated by the proton chemical shift. The delay $2\delta_1$ is used for refocusing the ^{13}C magnetization, which is antiphase with respect to the ^1H from which it originates. During this refocusing delay and the t_2 evolution period ^{13}C magnetization becomes antiphase with respect to its ^{13}C neighbor(s). The subsequent 90_x pulse serves a similar function as the 90° mixing pulse in a regular COSY experiment, transferring ^{13}C magnetization to its coupling partner(s). After a subsequent refocusing delay ($2\delta_2 + 2\Delta$), chosen on the order of $1/(4J_{CC})$ (which includes a $2\delta_2$ J_{CH} dephasing delay), an INEPT sequence[5] is used to transfer polarization back to the protons. To avoid sensitivity and resolution loss caused by the relatively large (~55 Hz) Cα—C=O coupling, homonuclear GARP decoupling[6] of the carbonyl resonances (using a very weak rf field, ~400 Hz) was used throughout the experiment. In the final 3D spectrum, the F_1 and F_3 coordinates of a resonance correspond to the origin and destination proton

(1) Fesik, S. W.; Zuiderweg, E. R. P. *J. Magn. Reson.* **1988**, *78*, 588. Marion, D.; Kay, L. E.; Sparks, S. W.; Torchia, D. A.; Bax, A. *J. Am. Chem. Soc.* **1989**, *111*, 1515. Zuiderweg, E. R. P.; Fesik, S. W. *Biochemistry* **1989**, *28*, 2387. Marion, D.; Driscoll, P. C.; Kay, L. E.; Wingfield, P. T.; Bax, A.; Gronenborn, A. M.; Clore, G. M. *Biochemistry* **1989**, *28*, 6150.
(2) Kainosho, M.; Tsuji, T. *Biochemistry* **1982**, *21*, 6273. McIntosh, L. P.; Griffey, R. H.; Muchmore, D. C.; Nielson, C. P.; Redfield, A. G.; Dahlquist, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1244. LeMaster, D. M.; Richards, F. M. *Biochemistry* **1988**, *27*, 142. Torchia, D. A.; Sparks, S. W.; Bax, A. *Biochemistry* **1989**, *28*, 5509.
(3) Oh, B. H.; Westler, W. M.; Darba, P.; Markley, J. L. *Science* **1988**, *240*, 857. Westler, W. M.; Kainosho, M.; Nagao, H.; Tomonaga, N.; Markley, J. L. *J. Am. Chem. Soc.* **1988**, *110*, 4093.
(4) Maudsley, A. A.; Muller, L.; Ernst, R. R. *J. Magn. Reson.* **1977**, *28*, 463. Bodenhausen, G.; Freeman, R. *J. Magn. Reson.* **1977**, *28*, 471.
(5) Morris, G. A.; Freeman, R. *J. Am. Chem. Soc.* **1979**, *101*, 760. Burum, D. P.; Ernst, R. R. *J. Magn. Reson.* **1980**, *39*, 163.
(6) Shaka, A. J.; Barker, P. B.; Freeman, R. *J. Magn. Reson.* **1985**, *64*, 547.

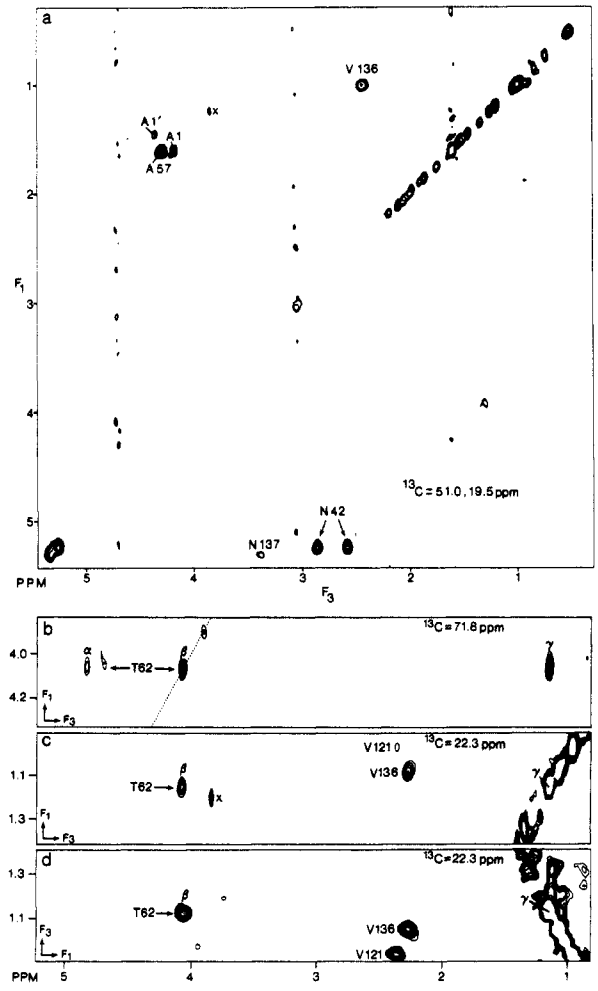

Figure 2. Slices from the 3D HCCH J correlation spectra of a 1.5 mM sample of the protein calmodulin, uniformly labeled (>90%) with ^{13}C and ^{15}N. Spectra a–c are obtained with the scheme of Figure 1; the section shown in d results from a 3D experiment with the central part of the pulse sequence (between the pair of simultaneous ^{1}H,^{13}C 90° pulses) reversed. Spectra result from a (64 complex) × (32 complex) × (512 real) data matrix, with acquisition times of 24, 8.1, and 52 ms in the t_1, t_2, and t_3 dimensions. After zero filling, the digital resolution is 10.4 Hz (F_1), 62 Hz (F_2), and 4.8 Hz (F_3). Because the presence of ^{13}C significantly shortens the ^{1}H T_1 relaxation time, only a short delay time (650 ms) was used between scans, resulting in a total measuring time of 50 h. The resonance A1′ in Figure 2a originates from protein molecules with an additional methionine residue at the N-terminus.

chemical shifts. The F_2 coordinate is the ^{13}C chemical shift of the carbon attached to the proton from which magnetization originates. The scheme of Figure 1 can also easily be changed to yield the ^{13}C shift of the destination carbon along the F_2 axis, providing complementary information.

Both versions of the 3D experiment were applied to calmodulin (16.7 kDa), uniformly (>90%) labeled with ^{13}C and ^{15}N, 1.5 mM in 99.9% D_2O, p^{2}H 6.3, 7 mM Ca^{2+}, 37 °C. Spectra were recorded on a modified Bruker AM-500 spectrometer. Extensive folding[7] of the ^{13}C frequencies was used in the F_2 dimension to yield higher digital resolution. For any given narrow proton frequency region the ^{13}C chemical shift range of carbons attached to these protons never exceeds 30 ppm. This allowed us to use an F_2 spectral width of only 31.5 ppm, without introducing any overlap in the (F_1,F_3) planes between folded and nonfolded resonances. Hence, for every slice two ^{13}C frequencies apply; from the ^{1}H frequency of the diagonal resonance it is clear which of the two ^{13}C frequencies is appropriate.

Figure 2a shows a (F_1,F_3) slice of the 3D spectrum taken at a F_2 frequency that corresponds to 51.0 and 19.5 ppm. Figure 2a shows dramatic simplification when compared with the ^{1}H COSY spectrum of unlabeled calmodulin (recorded at 600 MHz, supplementary material). Several cross peaks are found in the 3D spectrum that fall in well-resolved regions of the COSY spectrum but are too weak to be observed in the 2D map (for example, the Asn-137 CαH/CβH$_2$ cross peak seen in Figure 2a). The F_1,F_3 slice is asymmetric about the diagonal since only magnetization that originated from protons attached to carbons at 51.0 or 19.5 ppm is visible. The corresponding cross peak at the other side of the $F_1 = F_3$ diagonal is found in the slice taken at the ^{13}C frequency of the destination carbon. This is illustrated in Figure 2b,c for the side chain of Thr-62. The slice taken at the Cβ ^{13}C shift (Figure 2b) correlates CβH with both CαH and CγH$_3$ protons and the slice at the Cγ shift (Figure 2c) shows connectivity to the CβH proton.

As mentioned above, (F_1,F_3) slices of the 3D spectrum recorded with the scheme of Figure 1 are asymmetric about the diagonal and cross peaks appear in the F_3 dimension only (Figure 2a–c). By recording a 3D spectrum with a pulse sequence in which the central part (between the simultaneous 90° ^{1}H/^{13}C pulses) has been reversed, a 3D spectrum is obtained in which (F_1,F_3) slices show cross peaks in the F_1 dimension only. Figure 2d shows the "mirror image" of Figure 2c, recorded with such a reversed pulse sequence. Note that to minimize printing space requirements, the orientation of this section is rotated by 90° relative to Figure 2c.

Complete analysis of the 3D experiment described above yields unique spin system assignments for all non-aromatic residues. A combination of the HCCH 3D correlation spectrum with the 3D HMQC–NOESY spectrum[8] is particularly powerful, permitting many NOEs to be assigned uniquely in the first round of data analysis. This greatly facilitates the structure determination process.

The HCCH J correlation method solves the ^{1}H line width problem that hitherto has limited the size of proteins for which complete side chain assignments could be obtained. With the HCCH method, the ^{13}C line width is the major limiting factor. For ^{13}C resonances in calmodulin (17 kDa) the line widths vary from about 15 Hz for Cα protons to about 25 Hz for methylene carbons. Narrower line widths are found for many of the methyl groups. Since these line widths are still significantly smaller than the J_{CC} couplings, we expect that the methodology presented here will be efficient for proteins up to at least 25 kDa.

Of course, the 3D experiment can also be recorded in the 2D mode, albeit at the cost of resolution. However, well-known heteronuclear editing procedures can be used to simplify such spectra, making this approach a possibility for laboratories not yet set up for 3D NMR. The 3D spectrum required 50 h of measuring time and a relatively small 4 megaword data matrix before processing, indicating that this approach does not put any extraordinary requirements on instrument time or disk storage space while providing tremendous spectral simplification.

Acknowledgment. We thank Dr. Claude Klee for stimulating discussions and continuous encouragement, Marie Krinks for assistance in preparing the sample of calmodulin used in this study, Dr. Kathy Beckingham for providing us with the Drosophila calmodulin coding construct, and Drs. Paul Driscoll and Ingrid Pufahl for valuable comments during the preparation of the manuscript. This work was supported by the Intramural AIDS Antiviral Program of the Office of the Director of the National Institutes of Health. L.E.K. acknowledges financial support from the Medical Research Council of Canada and the Alberta Heritage Trust Foundation.

Supplementary Material Available: Figure giving one 600-MHz P. COSY spectrum of calmodulin (2 pages). Ordering information is given on any current masthead page.

(7) Kay, L. E.; Marion, D.; Bax, A. *J. Magn. Reson.* **1989**, *84*, 72.
(8) Ikura, M.; Kay, L. E.; Bax, A. *J. Magn. Reson.* In press.
(9) Bax, A. *J. Magn. Reson.* **1983**, *53*, 149.
(10) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393.