Table I. Comparison of Half-Times for [^{14}C]Cholesterol and [^{14}C]Sitosterol Exchange between Vesicles Prepared with Different Phospholipid Composition[a]

phospholipid composition	$t_{1/2}$, min cholesterol	$t_{1/2}$, min sitosterol
dipalmitoyl-PC	222.3 ± 43.2	990.8 ± 76.0
egg PC	14.9 ± 1.3	109.1 ± 12.7
egg PC/egg sphingomyelin	71.6	419.2

[a] The exchange experiments were carried out at 50 °C for dipalmitoyl-PC and egg PC vesicles and at 45 °C for egg PC (44 mol %)/egg sphingomyelin (35 mol %) vesicles. The sterol content was 1 mol % for dipalmitoyl-PC and egg PC vesicles and 6 mol % for egg PC/egg sphingomyelin vesicles. At least two different vesicle preparations were used in the experiments with dipalmitoyl-PC and egg PC. The sources and purities of the lipids have been cited elsewhere.[19,26] Photon correlation spectroscopy was used to estimate donor vesicle sizes at 50 °C. Measurements were made at a 90° scattering angle by using a 15-mV He-Ne laser of wavelength 632.8 nm together with a Brookhaven Instruments Corp. (Holtsville, NY) Model Bi-2030AT 128-channel digital correlator. A temperature-controlled scattering cell holder was used to maintain the samples at 50.0 ± 0.1 °C. The diameters of our sitosterol- and cholesterol-containing preparations were very similar. For dipalmitoyl-PC vesicles with 1 mol % sterol: sitosterol, 177-196 nm (index of polydispersity, 0.17-0.23); cholesterol, 245-264 nm (index of polydispersity, 0.23-0.29). For egg PC/egg sphingomyelin vesicles with 6 mol % sterol: sitosterol, 208 nm (index of polydispersity, 0.20); cholesterol, 206-240 nm (index of polydispersity, 0.19-0.25). Differential scanning calorimetry (Hart Scientific, Provo, UT) was used at a scanning rate of 15 °C/h to determine the phase-transition temperature of aqueous dispersions of dipalmitoyl-PC with 1 mol % sterol; no difference was found in the sitosterol- and cholesterol-containing preparations.

The kinetics of sterol exchange between dipalmitoyl-PC vesicles was examined over a prolonged period of time, appearing to reach equilibrium after about 60 h (Figure 1A). The exchange of radiolabeled sterol from negatively charged donor vesicles to a 10-fold excess of neutral acceptor vesicles was monitored as described previously.[19] The half-times for sterol exchange are more than 4-fold higher for sitosterol than for cholesterol in dipalmitoyl-PC bilayers (Table I). In egg PC bilayers (Figure 1B), the rates of sterol exchange are much faster than in dipalmitoyl-PC bilayers; this has been observed for cholesterol by several investigators[17,18a,19,20] and is considered to reflect the increased lateral packing density at the lipid-water interface in saturated relative to unsaturated PC bilayers. Table I shows that the half-time for sitosterol exchange is about 7 times higher than that for cholesterol exchange in egg PC bilayers. Incorporation of egg sphingomyelin (35 mol %) into egg PC bilayers results in a decrease in the rate of sterol exchange, with sitosterol undergoing exchange 6-fold more slowly than cholesterol.[21] The ability of sphingomyelin to lower the cholesterol exchange rate in mixed PC-sphingomyelin and sphingomyelin bilayers has been reported previously[20,22] and is explained by the greater lateral packing density in the lipid-water interface, which decreases the rate of cholesterol desorption. The magnitude of the difference in exchange rates between sitosterol and cholesterol was unexpected, since only a small difference in the promotion of acyl-chain order was noted in previous comparisons of their effects on phospholipid bilayers and monolayers,[23] furthermore, a membrane-*disordering* effect of sitosterol has been proposed in yeast.[24]

We suggest that the constraint in the intermembrane movement of sitosterol compared with cholesterol observed in our studies of exchange rates could account for *some* of the 5-10-fold-lower rate of absorption of sitosterol by intestinal mucosal cells. The results reported here support the model of the transition state postulated for cholesterol transfer between membranes, in which the cholesterol molecule is proposed to be attached by the tip of its hydrophobic side chain.[18a] The additional van der Waals interactions[25] between the sitosterol side chain and acyl chains of phospholipids appear to contribute significantly to the basis of discrimination in absorption of cholesterol and sitosterol. Future work will seek to evaluate whether the sterol to phospholipid molar ratio is an important factor in determining the relative absorptive discrimination of sterols. Comparative studies with ^{14}C-labeled sitosterol and cholesterol in biological membranes as the donor species would be worthwhile for evaluating the role of proteins in the transfer of these sterols between membrane surfaces.

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Registry No. Cholesterol, 57-88-5; β-sitosterol, 83-46-5; dipalmitoyl-PC, 2644-64-6.

Supplementary Material Available: A figure showing the kinetics of exchange of [^{14}C]cholesterol and [^{14}C]sitosterol from egg PC (44 mol %)/egg sphingomyelin (35 mol %)/dicetyl phosphate (15 mol %)/sterol (6 mol %) donor vesicles to a 10-fold excess of egg PC (94 mol %)/sitosterol (6 mol %) acceptor vesicles (1 page). Ordering information is given on any current masthead page.

(19) Fugler, L.; Clejan, S.; Bittman, R. *J. Biol. Chem.* **1985**, *260*, 4098-4102.
(20) Lund-Katz, S.; Laboda, H. M.; McLean, L. R.; Phillips, M. C. *Biochemistry* **1988**, *27*, 3416-3423.
(21) A figure of the time course of exchange of [^{14}C]cholesterol and [^{14}C]sitosterol from egg PC (44 mol %)/egg sphingomyelin (35 mol %)/sterol (6 mol %) vesicles to egg PC (94 mol %)/sitosterol (6 mol %) vesicles at 45 °C is presented as supplementary material.
(22) (a) Clejan, S.; Bittman, R. *J. Biol. Chem.* **1984**, *259*, 10823-10826. (b) Bhuvaneswaran, C.; Mitropoulos, K. A. *Biochem. J.* **1986**, *238*, 647-652. (c) Thomas, P. D.; Poznansky, M. J. *Biochem. J.* **1988**, *251*, 55-61.
(23) (a) Bittman, R.; Leventhal, A. M.; Karp, S.; Blau, L.; Tremblay, P.-A.; Kates, M. *Chem. Phys. Lipids* **1981**, *28*, 323-335. (b) Ghosh, D.; Tinoco, J. *Biochim. Biophys. Acta* **1972**, *266*, 41-49.
(24) Bloch, K. E. *CRC Crit. Rev. Biochem.* **1983**, *14*, 47-92.
(25) Reorientation of the steroid nucleus in PC bilayers caused by addition of methylene groups to an ester side chain at C_{21} of cortisone has been postulated to account for increased partitioning of C_{21}-substituted steroids into bilayers: Arrowsmith, M.; Hadgraft, J.; Kellaway, I. W. *Biochim. Biophys. Acta* **1983**, *750*, 149-156.
(26) Clejan, S.; Bittman, R. *J. Biol. Chem.* **1984**, *259*, 449-455.

2D and 3D NMR Spectroscopy Employing ^{13}C-^{13}C Magnetization Transfer by Isotropic Mixing. Spin System Identification in Large Proteins

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For large proteins (>10 kDa), proton NMR signals are difficult to assign due to the overlap of a large number of broad resonances.[1] The large line widths not only decrease the sensitivity and resolution but limit the number of correlations that can be obtained from 2D *J*-correlated experiments, since a rapid decay of proton magnetization occurs during the relatively long time required for coherence transfers involving small proton-proton *J* couplings.

In this communication, we describe a group of novel 2D and 3D NMR experiments that rely on magnetization transfer between

(1) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

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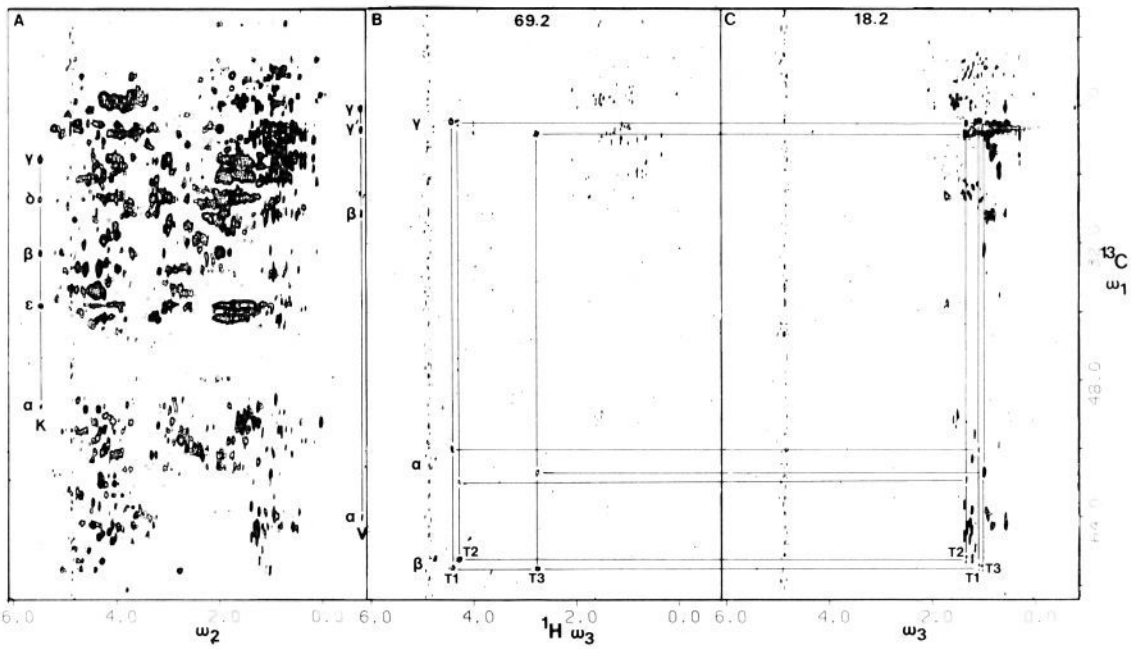

Figure 1. (A) Two-dimensional ^{13}C TOCSY–REVINEPT spectrum acquired on [^{13}C,^{15}N] T4 lysozyme with a MLEV-17 mixing period of 15.3 ms and a 1 ms trim pulse before and after the MLEV-17 sequence. (B and C) Individual planes of a 3D ^{13}C TOCSY–REVINEPT experiment acquired with an MLEV-17 mixing period of 15.3 ms at the ^{13}C frequencies (ω_2) shown at the top of the spectra. The vertical lines indicate the ^{13}C chemical shifts of different threonine spin systems. All NMR spectra were acquired on a Bruker AM500 NMR spectrometer at 20 °C with use of a 5-mm $^{13}C/^1H$ dual probe. A BSV3 CW amplifier connected to the observe channel was used for the hard 90° ^{13}C pulses (24 μs) and the MLEV-17 mixing scheme. An additional BSV3 driven from a PTS synthesizer and interfaced to a Smart pulse decoupler (Probe Systems) was used for ^{13}C decoupling with WALTZ-16.[8] The 2D experiment [$128(t_1) \times 2048(t_2)$] was collected in 11 h using 64 scans and 4 dummy acquisitions. To enhance the heteronuclear $^{13}C/^1H$ NOE and suppress the residual solvent signal, a series of 90° ^1H pulses separated by 10-ms delays was applied during the delay between scans. The 3D experiment was collected as a series of 54 complex (t_2) 2D experiments [$160(t_1) \times 2048(t_3)$] with use of a spectral width of ±4310 Hz in ω_1 and ω_2 and ±5681 Hz in ω_3 (oversampled).[9] Eight acquisitions and four dummy acquisitions were collected per t_3 experiment for a total experimental time of 262 h of which only half was spent on actual data acquisition due to instrumental limitations. All spectra were processed in the format of the FTNMR program of Dr. D. R. Hare with in-house written software utilizing a CSPI minimap array processor interfaced to a Vax 8350 computer.

^{13}C nuclei by isotropic mixing[2] and transfer of this information to the protons. Since *all* of the coherence transfers in the experiments can be carried out in a short period of time because of the large J couplings (≥35 Hz), the methods are applicable to large molecules with short relaxation times and can be used to correlate nuclei several bonds apart. The approach is illustrated by using a 3.8 mM sample of T4 lysozyme (MW = 19.7 kDa, 164 amino acids) that was uniformly labeled (>97%) with ^{13}C and ^{15}N.[3]

The key component in these experiments is a ^{13}C–^{13}C isotropic mixing step. Analogous to total correlation spectroscopy (TOCSY)[2a] or homonuclear Hartmann–Hahn (HOHAHA)[2b] experiments applied to protons, net ^{13}C coherence transfer occurs across multiple carbon–carbon bonds (in highly enriched molecules) and absorption mode peak line shapes are present, resulting in high sensitivity and enhanced resolution. As a ^{13}C–^{13}C correlation experiment, ^{13}C TOCSY or ^{13}C HOHAHA has several advantages over the ^{13}C double quantum correlation experiment,[4] including the possibility of resolving ambiguities in the ^{13}C NMR assignments from relay peaks.

In order to transfer the information derived from the ^{13}C–^{13}C TOCSY to the protons in a single experiment (and increase sensitivity), a ^{13}C–^1H correlation step is added to the ^{13}C TOCSY experiment to yield a heteronuclear ^{13}C–^{13}C–^1H relay experiment. For this purpose, we use the pulse sequence

$$90_\theta(^{13}C)\text{–}t_{1/2}\text{–}180_x(^1H)\text{–}t_{1/2}\text{–MLEV-17}(^{13}C)\text{–}\tau\text{–}180_x(^1H,^{13}C)\text{–}\tau\text{–}90_y(^{13}C),90_\phi(^1H)\text{–}\tau'\text{–}180_x(^1H,^{13}C)\text{–}\tau'\text{–acquire}(t_2)$$

in which $\theta = x, x, -x, -x$, $\phi = x, -x, x, -x$, and the receiver is cycled +, –, –, +. Basically, the sequence consists of a ^{13}C–^{13}C magnetization transfer using a MLEV-17 sequence[2b] followed by a heteronuclear ^{13}C–^1H transfer through a reverse INEPT experiment[5] and can therefore be called 2D ^{13}C TOCSY–REVINEPT. Since only large scalar couplings are utilized in these experiments for the coherence transfer, scalar connectivities can be traced across several bonds even in large molecules.

Figure 1A depicts a 2D ^{13}C TOCSY–REVINEPT spectrum of ^{13}C (and ^{15}N) labeled T4 lysozyme. Many additional relay cross-peaks are detected compared to a $^{13}C/^1H$ correlation spectrum (not shown) from which $^{13}C/^1H$ correlations across multiple bonds (e.g., Lys $\epsilon C/\alpha H$, Val $\alpha C/\gamma H$)[6] are obtained for this large protein.

Although some spin systems can be identified from the 2D spectra, the interpretation is difficult for more crowded regions. A dramatic improvement in resolution is obtained by extending this technique to a heteronuclear three-dimensional NMR experiment[7] in which an additional evolution period (t_2) is added

(2) (a) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521–528. (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.

(3) [^{13}C,^{15}N] T4 lysozyme was obtained from an inducible plasmid expression system in a prototrophic E. coli host grown in a 1-L minimal medium containing 3.0 g of $^{13}C_6$ glucose (99g isotopic purity, Cambridge Isotope Laboratories) as a carbon source and 1.7 g ($^{15}NH_4)_2SO_4$ (98.7%, Monsanto Mound) as a nitrogen source.

(4) (a) Westler, W. M.; Kainosho, M.; Nagao, H.; Tomonaga, N.; Markley, J. L. *J. Am. Chem. Soc.* **1988**, *110*, 4093–4095. (b) Stockman, B. J.; Westler, W. M.; Darba, P.; Markley, J. L. *J. Am. Chem. Soc.* **1988**, *110*, 4095–4096. (c) Bax, A.; Freeman, R.; Frenkiel, T. A.; Levitt, M. H. *J. Magn. Reson.* **1981**, *43*, 478–483.

(5) (a) Bendall, M. R.; Pegg, D. T.; Doddrell, D. M. *J. Magn. Reson.* **1981**, *45*, 8–29. (b) Freeman, R.; Mareci, T. H.; Morris, G. A. *J. Magn. Reson.* **1981**, *42*, 341–345.

(6) Since one-bond ^{13}C–^{13}C couplings are uniform, the peak intensities are predictable as a function of the isotropic mixing time, facilitating the assignments of the different amino acids. However, some of the assignments are difficult to obtain due to the limited bandwidth (~8 KHz) of the isotropic mixing process, restricting coherence transfers to only a small spectral region (in this case the aliphatic region).

(7) Fesik, S. W.; Zuiderweg, E. R. P. *J. Magn. Reson.* **1988**, *78*, 588–593.

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.

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(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

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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.

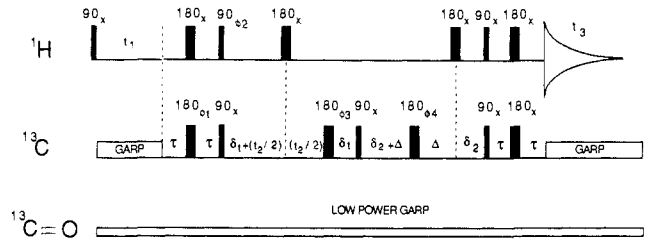

Figure 1. Pulse scheme of the 3D HCCH correlation technique. The delay τ is slightly shorter than $1/(4J_{CH})$, 1.5 ms. The delays δ_1 and δ_2 are $1/(6J_{CH})$ to permit optimal transfer from CH, CH_2, and CH_3 groups simultaneously. By setting δ_1 or δ_2 to a longer value, (CH)/(CH_2,CH_3) editing 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), $\phi_2 = y, -y$, ϕ_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.